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Investigating the Role of the Circadian Clock in the Plant Temperature Stress Response

A Dissertation submitted in partial satisfaction
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by

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Dedication

To my husband, Gabriel Jacobs, my family, and all the women in my life that inspire me

ABSTRACT OF THE DISSERTATION

Investigating the Role of the Circadian Clock in the Plant Temperature Stress Response

by

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As we consider the negative impacts of climate change-fueled crop loss with an increasing human population, it is critical that we understand how plants respond to environmental stresses. The circadian clock, which is a key regulatory unit that controls plant growth, metabolism, and physiology in concert with external stimuli, presents a compelling target to study plant stress response mechanisms. Here, I investigate how the circadian clock regulates the plant temperature stress response in *Arabidopsis*. First, a transcriptomic approach using clock mutants, *cca1lhy* and *ppr7prr9*, temperature stress, and two time points as variables revealed that the magnitude and occurrence of the *Arabidopsis* transcriptional response to heat stress is dependent on the time of day that the stress is applied. The transcriptome analysis culminated in the identification of ~200 clock regulated or time of day dependent genes including the cold-responsive, *CDF6*, and heat-responsive, *PLATZ2*, transcription factors. Second, characterization of *CDF6* demonstrated that *CDF6* is gated by the clock during cold stress. Vasculature expressed *CDF6* impacts germination during ambient temperature and photoperiodic flowering through downregulation of *FT, CO*, and *BFT* at ambient temperature and to a greater extent during cold stress. Third, examination of the *PLATZ* family of transcription factors determined that about half of the *PLATZ* family members exhibit rhythmic oscillation and respond to heat stress. *PLATZ2*

heat induction is dependent on the time of day the heat stress occurs, and it may contribute to the *Arabidopsis* response to high temperature. *PLATZ* orthologs in sorghum (*Sorghum bicolor*) and rice (*Oryza sativa*) display cyclic pattern of expression and one sorghum ortholog is also heat-responsive suggesting that the clock regulation of temperature stress response through the *PLATZ* family may be conserved among other species. To conclude, this work provides both an overview of clock regulation of the temperature stress response and a directed investigation of clock controlled genes during temperature stress. Overall, this work highlights the importance of considering the effect of both time of day and the circadian clock to understand how the clock adjusts to changing environmental conditions.

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Introduction

Climate change continues to result in increasing global temperatures, altered rainfall, and increased rate of extreme climate events, which in conjunction with the projected 2 billion increase in global population by mid-century threatens global food security (Godfray et al., 2010; Wheeler and von Braun, 2013; World Health Organization, 2021). Even before these major problems fully manifest, both moderate and severe food insecurity are already increasing globally with ~30% of the world population experiencing food security in 2020 (Wheeler and von Braun, 2013; World Health Organization, 2021). As a result of climate and population-fueled demand, scientists expect a global decrease in grain yield for critical caloric food sources including maize, rice, wheat, and soybean (Zhao et al., 2017). In rice specifically, yield is reduced by 3.2% per 1°C increase in global mean temperature (Zhao et al., 2017). Recent work to increase yield of major cereal crops still lags below the expected demand by 2050 (Ray et al., 2013). Therefore, to combat food insecurity, there is an urgent need in the scientific community to research plant mechanisms for stress tolerance.

Understanding how plants respond to extreme or stressful conditions can improve our ability to grow crops more efficiently in an unpredictable climate. Genetic engineering is an important tool to apply scientific findings to promote plant survival during extreme conditions. There are many examples of how genetic engineering has had and is predicted to have an impact on food security. For example, plant promoters can be used to engineer in a constitutive, tissue-specific, or inducible manner (Kummari et al., 2020). A combination of genetic strategies including trait pyramiding of genes that confer increased yield and stress resilience amongst many others has been extensively reviewed previously (Bailey-Serres et al., 2019). The National Academy of Sciences has also reported on the history, impacts, and future speculation of

genetically engineered crops (National Academies of Sciences, Engineering, and Medicine et al., 2017).

The plant circadian clock presents another interesting genetic engineering target due its role in regulating many agricultural traits including 30-50% of genes that respond to abiotic stress in *Arabidopsis thaliana* (*Arabidopsis*), a classical model for clock research (Bendix et al., 2015; Grundy et al., 2015; Steed et al., 2021). The circadian clock is an internal oscillator with a 24-hour period, which results from the Earth's rotation on its axis (Creux and Harmer, 2019). The clock was originally observed in the "sensitive heliotrope" (likely *Mimosa pudica)* when daily leaf movement was noted to continue in constant darkness (de Mairan, 1729) although the term "circadian" was not coined until much later in 1959 (Chandrashekaran, 1998; Halberg, 1959). The history of circadian clock discoveries has been previously and thoroughly reviewed (McClung, 2006). The complex circadian regulatory network enables organisms to synchronize their metabolism, physiology, and development to daily and seasonal environmental changes (McClung, 2006). Specifically, the clock can anticipate and adapt to changes in environmental stimuli (Bonnot et al., 2021). The clock requires input, typically light and temperature, from the external environment to allow synchronization with the internal oscillator in a process referred to as entrainment (Jones, 2009). The clock outputs biological rhythms evident in a variety of physiological processes including photosynthesis, flowering, response to abiotic stress, etc. (Nagel and Kay, 2012; Grundy et al., 2015; Nagel et al., 2015). The clock also allows organisms to maintain tissue-specific rhythms and is present in plants, animals, and some bacteria (Greenham and McClung, 2015; Endo, 2016; Markham and Greenham, 2021).

At the core of the plant circadian clock network are *CCA1 (CIRCADIAN CLOCK ASSOCIATED)* and *LHY (LATE ELONGATED HYPOCOTYL)* (Alabadí et al., 2001; Farré et al.,

2005; Nakamichi et al., 2010; Adams et al., 2015). CCA1 and LHY control the expression of a large number of target genes, ~2000 and ~700 genes respectively, including the *PSEUDO RESPONSE REGULATORS* (*PRRs*) (Alabadí et al., 2001; Farré et al., 2005; Nakamichi et al., 2010; Adams et al., 2015, 2018; Nagel et al., 2015). The *PRRs*, including *PRR9, PRR7, PRR5, PRR3,* and *PRR1/TOC1 (TIMING OF CAB EXPRESSION 1),* are expressed sequentially from mid-morning to dusk (Farré and Liu, 2013; Mody et al., 2020). As transcriptional regulators, the PRRs form negative feedback loops with the morning-expressed transcription factors, CCA1 and LHY (Nagel and Kay, 2012). *CCA1* and *LHY* form negative feedback loops with other evening expressed components including the evening complex (EC), which is comprised of *LUX ARRHYTHMO* (*LUX*), *EARLY FLOWERING 3* and *4* (*ELF3* and *ELF4*), and *GIGANTEA (GI)*, which regulates photoperiodic flowering through its association with light-stabilized *FLAVIN BINDING KELCH F-BOX 1* (*FKF1*) to ultimately control the expression of the floral promoters, *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) (McClung, 2006; Nagel and Kay, 2012). By controlling the transcription of these core oscillator components, in addition to many other core and downstream genes, the clock is able to precisely control timing of peak gene expression and thus output biological rhythms (Nagel and Kay, 2012; Mody et al., 2020). Many of these clock components are present across plant species, but have been primarily studied in *Arabidopsis* (Greenham and McClung, 2015).

Mutations in clock genes have a broad impact on output phenotypes. For example, both *cca1* and *lhy* loss of function mutants exhibit short periods, short hypocotyls, and early flowering, while the overexpression lines display arrhythmia, long hypocotyls, and late flowering (Nagel and Kay, 2012). Additionally, clock mutants often have impaired tolerance and acclimation to temperature stress and other abiotic stresses (Grundy et al., 2015). For example, *prr7* and *prr9*

mutants have increased freezing survival, while *cca1* and *lhy* mutants exhibit decreased freezing tolerance (Dong et al., 2011; Grundy et al., 2015).

Temperature serves an interesting role in the circadian clock in which temperature itself serves as an input, while the plant temperature stress response serves as an output. Additionally, the clock is also characterized by temperature compensation, a process in which biological rhythms are maintained across a broad range of physiologically relevant temperatures, which is somewhat counter-intuitive to general enzymatic kinetics principles (Avello Fernández et al., 2019). The molecular mechanisms of temperature compensation have been explored using the morning clock components, *CCA1, LHY, PRR9, PRR7*, and the evening component *GI*. Under high ambient temperature (27°C), *GI* and *LHY* balance each other to allow temperature compensation evidenced by impaired compensation in *gi* and *lhy* mutants (Gould et al., 2006). Under cold conditions (12°C), compensation is regulated by *CCA1* and *GI* (Gould et al., 2006*)*. Other morning clock components, *PRR9* and *PRR7*, also have a role in allowing temperature compensation (Salomé et al., 2010). In the *prr7prr9* double mutant, period length increases with temperature due to altered expression of *CCA1* and *LHY* evidenced by the rescue of the *prr7prr9* temperature overcompensation phenotype in the *cca1* and *lhy* knockdown lines (Salomé et al., 2010).

The plant temperature stress response is an important circadian output. The clock's regulation of the cold stress response has been well characterized through studying *C-REPEAT BINDING FACTOR 1, 2,* and *3* (*CBF1-3*). *CBF1, 2,* and *3* have redundant functional activity including in regulating freezing tolerance and gene expression (Gilmour et al., 2004). C-REPEAT BINDING FACTOR 1 (CBF1) was first identified as a transcriptional activator that binds to the C-repeat /drought-responsive element (CRT/DRE) which induces cold-responsive (*COR*) genes

to promote freezing tolerance (Jaglo-Ottosen et al., 1998). Previous work identified ~300 *COR* genes, which were mostly upregulated during cold stress (Fowler et al., 2005; Shi et al., 2017). More recently, transcriptomic analysis identified ~3000 *COR* genes using *cbf123,* a triple loss of function mutant, and found that the *CBFs* coregulate the majority *COR* genes, but approximately one-third are regulated by a single *CBF* (Shi et al., 2017). The *CBFs* accumulate in response to cold, but that accumulation is dependent on the time of day that the cold stress occurs (Fowler et al., 2005). *CBF* peak accumulation occurs after the cold stress is applied at Zeitgeber Time 4 (ZT4), or four hours after illumination (Fowler et al., 2005). Conversely, the trough of accumulation occurs when the cold stress is applied at ZT16 (Fowler et al., 2005). This striking time of day difference suggests circadian regulation of the *CBFs*, thus, *CBF* accumulation during temporally-controlled cold exposure was evaluated in a *CCA1-OX* (*35S::CCA1*) background (Fowler et al., 2005). The overexpression of *CCA1* abolished the accumulation of the *CBFs* in response to the cold exposure at all times of day indicating that the circadian clock, through CCA1, gates (limits) the expression of the *CBFs* in response to cold stress (Fowler et al., 2005). Gating occurs when the same magnitude of a stress occurs at multiple times of day and results in a different molecular response. Further, the *CBFs* contain 19 copies of the Evening Element (EE; AAAATATCT), a cis-regulatory motif associated with circadian clock regulation through CCA1, and 35 copies of the CCA1 Binding Site (CBS; AATCT), another cis-element regulated by the clock through CCA1 (Dong et al., 2011). ChIP quantitative PCR (qPCR) analyses experimentally supports CCA1 binding to the promoter regions of CBFs (Dong et al., 2011). Additionally, the promoters of *COR* genes are enriched for the EE (Mikkelsen and Thomashow, 2009). Taken together, these data provide strong support for circadian control by the transcription factor CCA1, of the cold stress response by specifically regulating the expression of the *CBFs* and a subset of *COR* genes during cold stress.

In this dissertation, I aimed to further elucidate the functional role of the circadian clock in the plant temperature stress response. In my first objective, I identified transcriptome-wide dynamics of clock-controlled temperature stress responses in *Arabidopsis* using clock mutants (*cca1lhy* and *prr7prr9*) after a 1-hour temperature pulse of either 10°C or 37°C at time points relevant to transcriptional regulator expression (ZT0 for *cca1lhy* and ZT6 for *prr7prr9*). I found that both the magnitude and occurrence of the transcriptional response is highly dependent on the time of day that a temperature stress is applied. I also identified a subset of ~200 genes that require the circadian clock or time of day to respond to temperature, including the transcription factor genes *CDF6* and *PLATZ2*, which respond to cold and heat stress, respectively. In my second objective, I characterized the clock-regulation of *CDF6* and found that the clock gates the accumulation of *CDF6* mRNA during cold stress. I also determined that expression of *CDF6* in the vasculature (phloem companion cells) is sufficient to delay photoperiodic flowering and germination in a temperature dependent manner. RNA-sequencing and qPCR both confirmed that numerous flowering-associated genes, including *FT*, are negatively regulated by *CDF6*. In my third objective, I studied *PLATZ2* and other *PLATZ* transcription factors to determine their role in the plant heat stress response. I found that about half of the *Arabidopsis PLATZ* genes are clock regulated and respond to temperature. *PLAZ2* is induced by heat stress at certain times of day and may play a role in the heat stress response. I also found that one of the 11 sorghum *PLATZ* genes cycles and responds to heat, while ~50% of the rice *PLATZ* genes exhibit cyclic patterns of expression. In summary, my dissertation work identified clock controlled, temperature responsive genes, and it characterized both cold-responsive (*CDF6*) and heat-responsive (*PLATZ2*) genes to more closely examine how the clock regulates the plant temperature response to overall impact growth and development during stress.

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Chapter 1

Contribution of time of day and the circadian clock to the heat stress responsive transcriptome in *Arabidopsis*

Abstract

In *Arabidopsis*, a large subset of heat-responsive genes exhibit diurnal or circadian oscillations. However, to what extent the dimension of time and/or the circadian clock contribute to heat stress responses remains largely unknown. To determine the direct contribution of time of day and/or the clock to differential heat stress responses, we probed wild-type and mutants of the circadian clock genes *CCA1, LHY, PRR7,* and *PRR9* following exposure to heat (37ºC) and moderate cold (10ºC) in the early morning (ZT1) and afternoon (ZT6). Thousands of genes were differentially expressed in response to temperature, time of day, and/or the clock mutation. Approximately 30% more genes were differentially expressed in the afternoon compared to the morning, and heat stress significantly perturbed the transcriptome. Of the DEGs (~3000) specifically responsive to heat stress, ~ 70% showed time of day (ZT1 or ZT6) occurrence of the transcriptional response. For the DEGs (~1400) that are shared between ZT1 and ZT6, we observed changes to the magnitude of the transcriptional response. In addition, ~2% of all DEGs showed differential responses to temperature stress in the clock mutants. The findings in this study highlight a significant role for time of day in the heat stress responsive transcriptome, and the clock through *CCA1* and *LHY*, appears to have a more profound role than *PRR7* and *PRR9* in modulating heat stress responses during the day. Our results emphasize the importance of considering the dimension of time in studies on abiotic stress responses in *Arabidopsis*.

Introduction

The clock enables organisms to synchronize their metabolism, physiology, and development, to predictable daily and seasonal environmental changes conferring enhanced fitness and growth vigor in the plants (Dodd et al., 2005; Harmer and Kay, 2005; McClung, 2006; Nagel and Kay, 2012). Time of day information is gathered through key inputs such as light, temperature, and metabolite levels (Millar, 2004; Jones, 2009; Graf and Smith, 2011; Paparelli et al., 2013; Kromdijk et al., 2016). Underlying the clock network are multiple feedback loops, with interconnected components that interact both negatively and positively to influence a wide range of cellular and biological processes (Harmer and Kay, 2005; McClung, 2006; Pruneda-Paz and Kay, 2010; Helfer et al., 2011; Nusinow et al., 2011; Sanchez et al., 2011; Nagel and Kay, 2012; Nohales and Kay, 2016; Sanchez and Kay, 2016). At the core of the oscillator, two closely related Myb domain transcription factors (TFs), CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), both expressed in the morning, negatively regulate the expression of the evening phased clock gene *TIMING OF CAB EXPRESSION1 (TOC1*) (Alabadí et al., 2001; Gendron et al., 2012; Huang et al., 2012; Pokhilko et al., 2013). TOC1 in turn completes the loop by regulating *CCA1* and *LHY* expression in the late evening (Alabadí et al., 2001; Gendron et al., 2012; Huang et al., 2012; Pokhilko et al., 2013). Besides this core feedback loop, CCA1 and LHY regulate the expression of *PSEUDO RESPONSE REGULATOR 7 (PRR7)* and *PRR9*, which are expressed during the day as part of a morning regulatory loop (Farré et al., 2005; Nakamichi et al., 2010; Adams et al., 2015). These four components (*CCA1, LHY, PRR7* and *PRR9*), along with a few other evening expressed clock genes, are important for maintaining a relatively constant period (~ 24 h) within a range of growth permissive temperatures (~12ºC - 30ºC) (Salomé and McClung, 2005; Gould et al., 2006; Salomé et al., 2010).

Both temperature and the clock control many aspects of plant growth and fitness through extensive regulation of gene expression (Green et al., 2002; Michael et al., 2003; Dodd et al., 2005; Bita and Gerats, 2013). The clock also directly influences key crop traits while high temperature stress can alter crop productivity (Bendix et al., 2015; Zhao et al., 2017). Based on transcriptome experiments, up to 50% of the genes responsive to heat, cold, salinity, osmoticum, or water deprivation show circadian rhythmicity in *Arabidopsis* (Covington et al., 2008; Legnaioli et al., 2009; Greenham and McClung, 2015). Rhythmic expression of abiotic stress-responsive genes is also observed in soybean and barley (Habte et al., 2014; Marcolino-Gomes et al., 2014).

Mechanistically, the clock is able to regulate the expression of these stress responsive genes by controlling the magnitude or occurrence of the response based on time of day, a process referred to as gating (Harmer, 2009; Wilkins et al., 2009, 2010; Thomashow, 2010; Greenham and McClung, 2015; Pudasaini et al., 2017). The relevance of time of day transcriptomic changes for a given stress response such as drought or cold has been comprehensively examined and continues to emerge (Bieniawska et al., 2008; Wilkins et al., 2009, 2010; Thomashow, 2010; Calixto et al., 2018). For example, cold induction of the *C-REPEAT BINDING FACTORS* (*CBFs;* also known as *DEHYDRATION-RESPONSIVE ELEMENT-BINDING (DREB)* TF transcript levels in response to cold (4°C) is higher in the early morning (4 h after dawn) versus evening (16 h after dawn), and this response is altered in the *CCA1 (cca1-11)* and *LHY (lhy-21)* clock mutants (Thomashow, 1999; Fowler et al., 2005; Dong et al., 2011).

Numerous transcriptomic studies following various degrees of high temperature stress have been reported (Hruz et al., 2008; Frank et al., 2009; Liu et al., 2012; Zhang et al., 2012, 2013b; Kumar et al., 2015; Rawat et al., 2015; Wu et al., 2015; Chen and Li, 2016). However, most of these studies lacks information on the dimension of time, suggesting that some heat stress responsive genes might be overlooked. A recent targeted study, suggests a role for the evening

expressed clock components *TOC1* and *PRR5* in gating the molecular responses of select genes to warm temperature (high ambient temperature) (Zhu et al., 2016). However, a global understanding of the contribution of time of day and/or the clock to the high temperature responsive transcriptome during the day period when plants are exposed to maximum heat stress and likely primed for high temperature remains poorly understood (Grundy et al., 2015).

Therefore, to determine to what extent time of day and the circadian clock contribute to differential transcriptional responses under heat stress in *Arabidopsis*, we assayed for transcriptomic changes under temperature stress in the early morning (ZT1) and the early afternoon (ZT6); times when temperature stress responsive genes, and the morning and day expressed clock genes also exhibit peak expression (Grundy et al., 2015). From the thousands of genes that were differentially expressed in response to heat treatment and time of day (ZT1 vs ZT6), ~33% and ~38% are specific to either ZT1 or ZT6, respectively, while ~30% are shared between both time points. In all three categories, the majority (> 50%) of the DEGs were upregulated in response to heat stress, and the response is more evident in the early afternoon relative to the early morning. In addition, among all of the DEGs, 2% showed differential expression in response to temperature stress in the clock mutants and also when compared to wild-type. Our analyses have revealed that during the day when plants are exposed to maximum high temperatures, time of day plays an extensive and important role in modulating the heat stress responsive transcriptome, and this sensitivity is more evident in the early afternoon. In addition, the clock through the morning expressed clock genes *CCA1* and *LHY*, also modulate the heat stress responses preferentially in the early morning. In summary, this analysis provides the first global analysis on the contribution of time of day and/or the clock to heat stress responses in *Arabidopsis.*

Materials and Methods

Plant Materials and Growth Conditions

In all experiments, *Arabidopsis thaliana* Columbia-0 (Col-0) was used as wild-type (WT). The *cca1-1/lhy-20 (cca1lhy)* was generated by crossing the previously characterized *cca1-1* mutation in Col-0 with *lhy-20* also in Col-0 background (Green and Tobin, 1999; Michael et al., 2003). The *prr7-3/prr9-1 (prr7prr9)* in Col-0 was previously characterized (Farré et al., 2005; Pruneda-Paz et al., 2009). Seeds were surface sterilized and stratified in the dark at 4°C for 3 days. Plants were grown on plates containing Murashige and Skoog (MS) medium supplemented with 1.5% sucrose (wt/vol) in 12 h light and 12 h dark (LD) cycles for 12 days at constant 22ºC and 90 µm light intensity. For the RNA-sequencing experiment, a subset of plants was transferred for 1 h to a growth chamber set to either 10ºC or 37ºC on day 13. For *cca1lhy,* plants were transferred at lights on (ZT0) and for *prr7prr9*, 5 h after lights on. Whole seedlings were collected one hour after temperature treatments corresponding to ZT1 and ZT6.

RNA Extraction and RNA-sequencing Library Preparation

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following manufacturer's protocol. The extracted RNA was DNaseI treated to remove contaminating DNA (Millipore Sigma, Burlington, MA, USA). Next between 2µg and 5µg of total RNA was mRNA purified with Dynabeads Oligo-dT(25) (Wang et al., 2011). Libraries were prepared as described previously (Kumar et al., 2012). Modifications to the protocol are as follows: for the adapter ligation, EDTA was not added to the samples during adapter ligation, and samples were always eluted from the beads using RNAse-free water instead of 10 mM Tris-HCl pH 8. In the final enrichment, Kapa HIFI (Kapa Biosystems, Wilmington, MA, USA) was used instead of Phusion Polymerase, and we performed 15 cycles to amplify the libraries. Final libraries were either purified

by Ampure XP beads or SDS-PAGE gel extraction (Juntawong et al., 2015; Townsley et al., 2015). Library concentration and quality were verified using a Qubit 2.0 Fluorescence Reader (ThermoFisher Scientific) and Bioanalyzer 2100 (Agilent Genomics). Libraries consisting of four biological replicates for Col-0 and *cca1lhy* after one hour treatment (ZT1), and two replicates of Col-0 and *prr7prr9* after one hour treatment (ZT6) for each temperature were multiplexed with unique barcodes and sequenced.

RNA-sequencing and Data Analysis

Single-end 75 base pair sequences were generated for each mRNA library using the NextSeq 500 (Illumina) at the UC Riverside (UCR) Institute for Integrated Genome Biology (IIGB) Genomics Core facility. Data analysis was conducted by following the systemPipeR workflow using a computer cluster operated by the UCR IIGB facility, specifically using R version 3.4.3(H Backman and Girke, 2016). To control quality of reads, we used cutadapt with default settings and FastQC reports (H Backman and Girke, 2016). Trimmed reads were mapped to the TAIR10 genome using the alignment software, TopHat2 (2.0.14) and Bowtie2 (2.2.5) (H Backman and Girke, 2016). Exons were counted by "union" mode with GenomicFeatures using the Araport11 gff (201606). An offset of 5 counts was applied to all counts followed by library scaling by edgeR.calcNormFactors (H Backman and Girke, 2016). Limma was employed to conduct differential gene expression analysis specifically using quantile normalization with voom (H Backman and Girke, 2016). Log-fold change (LFC) was calculated by subtracting the normalized CPM values between treatments, while false discovery rate (FDR) was calculated with the Benjamini & Hochberg method (H Backman and Girke, 2016). Differentially expressed genes (DEGs), used for downstream analysis and heatmap clustering, were selected by filtering for Log_2

Fold Change (LFC) > |1| and FDR < 0.05. Clustering for heatmaps utilized the Euclidean method with partition around medoids (H Backman and Girke, 2016).

Comparison with Other Temperature Stress Experiments

Genevestigator was used to identify heat and cold stress experiments performed in Col-0 plants (Hruz et al., 2008). The Genevestigator accession numbers used were: AT-00120, AT-00176, AT-00221, AT-00230, AT-00288, AT-00389, AT-00402, AT-00500, AT-00633, AT-00640, AT-00641, AT-00645, AT-00654, AT-00670, and AT-00751.

Quantitative Real-time PCR

Seeds were prepared as described above and grown in LD conditions at 22 °C for 12 d. Samples were collected every 4 h in LD and total RNA was isolated with the Qiagen RNeasy plant mini kit (Qiagen, Hilden, Germany). cDNA was synthesized using 1 µg of total RNA and was reverse-transcribed with the iScript cDNA synthesis kit (Bio-Rad). See Supplementary Table 1.S1 for gene specific primers and qRT-PCR conditions used here.

Interaction Network Analysis

To analyze interactions between selected TFs, published Chromatin Immunoprecipitation followed by deep sequencing (ChIP-seq) data for *CCA1, LHY, PRR7* and *PRR9*, and DNA Affinity Purification sequencing (DAP-seq) data for *HB21* were used (Liu et al., 2013, 2016; Nagel et al., 2015; O'Malley et al., 2016). *CCA1, LHY, PRR7, PRR9,* and *HB21* were defined as sources. Targets were restricted to differentially expressed transcription factors. Interaction networks were visualized using Cytoscape software 3.3.0 (Smoot et al., 2011).

Data Availability and Deposition

The sequences reported in this paper have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database and can be accessed through GEO Series accession GSE116004.

Results and Discussion

Time of day specific transcriptome changes in response to temperature stress

To first determine how the transcriptional response of clock genes to heat stress is altered, we examined changes in transcript abundance of *CCA1, LHY, PRR7*, and *PRR9*, clock genes that show peak expression throughout the day period (**Figure 1.1A**). Wild-type (WT) *Arabidopsis* seedlings were exposed to heat stress at two times of day, early morning and early afternoon (indicated in **Figure 1.1A**). These time-points were selected because they relatively correspond to the time of day when genes that are upregulated by heat or downregulated by cold show peak expression (dawn) and when the maximum heat stress responses occur (Grundy et al., 2015). We performed quantitative real-time PCR (qRT-PCR) on samples collected from seedlings grown in 12 h light/12 h dark cycles (LD) and constant temperature (22°C) for twelve days, then exposed for 1 hour to 37ºC on day thirteen at dawn or early afternoon (**Figure 1.1A, 1.1B**). We observe enhanced expression of *CCA1, PRR7*, and *PRR9* and a reduction in *LHY* expression under heat stress, confirming that these clock components are responsive to high temperature stress, and therefore, are likely to affect the transcriptional response of their downstream targets (**Figure 1.1B**). This is consistent with a recent transcriptomic report showing that these clock genes are similarly differentially expressed even after 30 mins of heat treatment (37°C) (Albihlal et al., 2018).

To assess the global gene expression dynamics between time of day, the clock, and heat stress responses, we performed RNA-sequencing on WT and mutant *Arabidopsis* seedlings of

CCA1, LHY, PRR7, and *PRR9,* using a similar heat treatment (1 h at 37°C) as described above, and included a moderate cold stress treatment (1 h at 10°C) to distinguish between heat responsive genes and general temperature responsive genes. We selected the double mutant lines for *CCA1* and *LHY* (*cca1-1/lhy-20*) since these two clock genes showed opposing heat responses, and *PRR7* and *PRR9* (*prr7-3/prr9-1*) to cover both the midday and early afternoon times of the day (Green and Tobin, 1999; Michael et al., 2003; Farré et al., 2005; Pruneda-Paz et al., 2009). Twelve day old WT, *cca1-1/lhy-20* (*cca1lhy*) and *prr7-3/prr9-1* (*prr7prr9*) seedlings were exposed for 1 h to 10°C or 37°C at dawn (ZT0, at lights ON) or early afternoon (ZT5, after lights ON) and collected at ZT1 or ZT6, respectively (Green and Tobin, 1999; Michael et al., 2003; Farré et al., 2005; Mockler et al., 2007). We filtered the resulting datasets for genes with a Log_2 Fold Change (LFC) > |1| and False Discovery Rate (FDR) < 0.05 to be more inclusive and to consider that even small differences in expression levels might have a significant impact on the regulation of some genes (**Supplementary Dataset 1.S1**). Based on these criteria, we obtained 6266 and 8183 DEGs that represent both the genotype and temperature condition at ZT1 and ZT6, respectively (**Figure 1.1C; Supplementary Dataset 1.S2**). Most of the known clock genes exhibit differential expression in response to temperature stress **(Supplementary Dataset 1.S2**) (Hsu and Harmer, 2014). Consistent with our qRT-PCR results in Figure 1.1B, *CCA1, PRR7,* and *PRR9* showed upregulation and *LHY* downregulation following heat stress (37°C) (**Figure 1.1D**). However, based on our FDR cut-off criteria, *CCA1* upregulation at 37°C is only significant at ZT6, while *LHY* downregulation is significant at ZT1 (**Figure 1.1D**). At 10°C, *CCA1* and *PRR9* appear to also be upregulated, while *LHY* and *PRR7* exhibit opposite expression, upregulated and downregulated, respectively (**Figure 1.1D**). Overall, more genes were differentially expressed at ZT6 vs ZT1, and although 4130 DEGs are shared between the ZT1 and ZT6 datasets, approximately 50% are specific to each dataset,

emphasizing the importance of time of day in transcriptomic analysis when assaying for temperature stress responses (**Figure 1.1C**).

As multiple temperature stress related genome-wide experiments have been performed, we compared our list of DEGs with a selected subset of expression datasets available through Genevestigator along with the most recently published heat and cold RNA-seq experiments at the time of this analysis (Hruz et al., 2008; Albihlal et al., 2018; Calixto et al., 2018). While most of our DEGs were shared with these other experiments, we identified DEGs that are specific to either our cold (2.77%) or heat stress (7.94%) DEGs (**Supplementary Figure 1.S1A, 1.S1B and Dataset S2**). Because we did not account for differences due to analysis pipeline, treatment duration, plant growth conditions, developmental stage, and all published or unavailable experiments, this comparison is not fully conclusive. Although the upregulation of *CCA1* at 37°C is not significant in our dataset at ZT1 in WT, it is significantly upregulated in the Albihlal *et al.,* 2018 dataset, where the growth conditions, duration of 37°C treatment, and analysis pipeline differ, and information on the time of day the treatment was applied, is unknown. Similarly, *TOC1* is significantly upregulated at 37°C in the Albihlal *et al.,* 2018 but is not differentially expressed in our WT dataset based on our selection criteria, highlighting the limitations of comparative analysis between multiple available data sources that are derived from different experimental conditions and analysis pipeline, etc (**Supplementary Dataset 1.S2**).

Majority of the DEGs show differential response to heat stress

From all the genotype and condition specific datasets, we first assessed the overall effects of temperature treatment in the context of time of day or the clock mutants on the transcriptome. In both the ZT1 and ZT6 datasets, the treatment at 37°C highly perturbed the transcriptome compared to 10°C in the WT and clock mutants (**Figure 1.2A and 1.2B; Supplementary Dataset 1.S3**). For

example, in the WT, while 3199 (ZT1) and 3359 (ZT6) genes showed differential expression at 37°C relative to 22°C, only 256 (ZT1) and 970 (ZT6) genes were differentially expressed at 10°C compared to 22°C in WT (**Supplementary Figure 1.S2A**). Similar numbers of genes were upregulated and downregulated at ZT1 (1771 and 1598) and ZT6 (1970 and 1787) in response to heat stress (37°C/22°C; **Supplementary Figure 1.S2B**). Consistent with this observation, enriched gene ontology (GO) functional categories include processes related to high temperature stress (response to heat and heat acclimation), as indicated in clusters 8 and 10 in the ZT1 dataset and clusters 6 and 9 for ZT6 dataset, and include many of the known *Heat Shock Transcription Factors (HSFs)* and *Heat Shock Proteins (HSPs)* (**Figure 1.2C and 2D; Supplementary Dataset 1.S4**) (Driedonks et al., 2015). In these clusters (8 and 10 for ZT1; 6 and 9 for ZT6), DEGs were primarily upregulated in response to heat stress. In addition to heat stress related categories, additional enriched categories include response to abscisic acid (ABA), alcohol and lipids, and transcription. DEGs downregulated in response to heat stress at ZT1, in clusters 2, 4, and 9, were mostly enriched in biological processes such as metabolic processes, chloroplast accumulation and movement, and sulfate reduction and assimilation (**Figure 1.2C and Supplementary Dataset 1.S4**). At ZT6, in addition to heat stress related processes, upregulated DEGs were also enriched for circadian rhythms, rhythmic process, and response to water deprivation, while down-regulated DEGs were enriched for metabolic processes, nucleosome organization and assembly, ribosomal large subunit biogenesis/assembly, and photosynthesis (**Figure 1.2D and Supplementary Dataset 1.S4**).

Because the *cca1lhy* mutant exhibits an earlier shift in peak gene expression and *prr7prr9* mutants a delayed shift in peak gene expression in light dark cycles, direct comparison between WT and mutants could lead to confounding results, specifically false positives, since differential expression at a single time-point could reflect a shift in expression rather than actual differences in peak expression in response to temperature stress between the genotypes (Mizoguchi et al., 2002;

Farré et al., 2005; Ding et al., 2007; Zhang et al., 2013a). However, we nevertheless wanted to determine whether the DEGs enriched in some clusters in WT or clock mutants at ZT1 and ZT6 in response to heat stress can be linked to clock function. We observed that only at ZT1, DEGs in cluster 3 that contained either up- or down-regulated genes in WT or *cca1lhy* mutant at 37°C, showed up-regulation in the *cca1lhy* mutant to WT comparison (**Figure 1.2A**). This cluster was enriched for GO terms such as circadian rhythm, rhythmic process, and response to cold, and most of the evening expressed circadian clock genes grouped within this cluster (**Figure 1.2C**).

Time of day contributes to differential transcriptional responses under heat stress

In nature, the early morning and early afternoon are times of the day when heat responsive genes are highest expressed. As described above, for both ZT1 and ZT6, a similar number of genes, 3199 (51%) and 3027 (48%) were differentially expressed in response to 37°C in the WT and in the *cca1lhy* mutant, and 3359 (41%) and 3432 (42%) DEGs in the WT and in the *prr7prr9* mutant, respectively (**Supplementary Figure 1.S2A and Dataset S2**). Therefore, we were interested to determine the extent to which the occurrence or magnitude of the transcriptional response to heat stress is modulated by the time of day the stress was applied (ZT1 vs ZT6). For this we compared the ZT1 (3369) and ZT6 (3757) DEGs in response to 37ºC in WT (**Supplementary Figure 1.S2A**). Approximately 50% are specific to either ZT1 (1742 DEGs) or ZT6 (1902 DEGs), suggesting that the occurrence of the response is specific to certain times of the day (**Supplementary Dataset 1.S5**). However, because some of these genes also show differential response to moderate cold stress (10°C), we considered these DEGs as general temperature stress responsive genes and thus excluded them from the heat specific analysis. We obtained 1606 DEGs specific to ZT1 and 1846 DEGs specific to ZT6, where the occurrence of the transcriptional response to heat stress depends on the time of day the treatment was applied (ZT1 vs ZT6; **Figure 1.3A and 1.3B**).

In terms of modulation of the response depending on time of day, ~30% (1457) of the DEGs were shared between ZT1 and ZT6, many of which showed time of day specific transcriptional changes (**Figure 1.3A and 3B; Supplementary Dataset 1.S5**). Most DEGs (~ 65%) are upregulated in response to 37°C and enriched for GO biological processes involved with heat stress such as, cellular response to chaperone mediation and unfolded protein, heat acclimation, and hydrogen peroxide, as expected (**Figure 1.3C and Supplementary Dataset 1.S4**). Although a lesser number of genes (~ 35%) that show differential expression between ZT1 and ZT6 at 37°C are downregulated, these DEGs are enriched for GO biological processes photosynthesis, response to light, cell wall modification, nucleosome assembly, and metabolic processes (**Figure 1.3C and Supplementary Dataset 1.S4**). Furthermore, ~2/3 (937/1457) of the transcriptional responses exhibit a greater induction (upregulated DEGs) or repression (downregulated DEGs) at ZT6 (**Supplementary Dataset 1.S5**). We further examined these shared time of day specific DEGs (1457) and found that ~ 8% (119) show either a LFC ratio > 2 or < 0.5 between the LFC at ZT1 and ZT6 with a greater LFC at ZT6 (**Figure 1.3D**). These 119 DEGs were not significantly enriched for any specific GO processes. However, several of these genes are characterized as hypothetical proteins of unknown function, and could be interesting candidates for follow-up studies on time of day control of heat stress responses (**Figure 1.3D**). It has recently been shown that alterations to the phase and period of clock and clock controlled genes in response to heat stress, occurs after ~12 h under constant exposure to relatively high temperature (35°C) (Gil et al., 2017). Therefore, we reasoned that differential expression of clock genes and clock output genes in response to acute heat stress at ZT1 and ZT6 after lights ON are most likely due to the time of the day the stress was applied.

Only a few DEGs (5 genes), mostly of unknown function, showed opposing response between ZT1 and ZT6 at 37ºC (**Figure 1.3B and 1.3C**). Of note, in the comparison between ZT1

and ZT6 for the DEGs specifically at 10°C in WT, we observed similar time of day specific transcriptional changes (**Supplementary Dataset 1.S5**). Reports that time of the day can modulate the magnitude of the transcriptional response to cold stress have been previously characterized (Fowler et al., 2005; Dong et al., 2011; Lee and Thomashow, 2012). In our analysis, we also observed that the upregulation of *CBF2 (AT4G25470)* in response to moderate cold stress (10°C) is greater at ZT6 (LFC 5.1; FDR 3.1^{E-03}) vs ZT1 (LFC 4.3; FDR 3.4^{E-07}).

We identified 13 *HSFs* that are differentially regulated by heat stress and six of them (*HSFA1D, HSFA1E, HSFA3, HSFA6B, HSFA7A,* and *HSF4*) showed modulation of the transcriptional response at ZT1 vs ZT6. For example, induction of *HSFA3* in response to heat stress is greater at ZT6 relative to ZT1 (LFC of 3.04 vs 1.85 and FDR of 3.0^{-03} vs 1.35^{-05}, respectively) in WT, suggesting that *HSFA3* is more sensitive to the heat stress in the early afternoon. Interestingly, the induction of *HSFA3* is also strongly enhanced in the *cca1lhy* (LFC 3.12; FDR 2.11^{-08}) and *prr7prr9* (LFC 3.63; FDR 0.001) mutants at 37°C relative to 22°C, suggesting that the clock might also play an important role in modulating the transcriptional response of *HSFA3* to high temperature stress during the day.

The clock controls the magnitude of the transcriptional response for a subset of DEGs

In *Arabidopsis*, up to 50% of the genes that are responsive to heat, cold, and other abiotic stresses show circadian rhythmicity (Harmer et al., 2000; Edwards et al., 2006; Covington et al., 2008; Michael et al., 2008; Legnaioli et al., 2009; Greenham and McClung, 2015). Our analysis was performed to determine the contribution and connection between time of day and the clock modulation of heat stress responses through identification of the genes underlying this regulation.

Overall, from the thousands of genes that were differentially regulated in our analysis either by time, treatment, or genotype, we obtained 213 genes that were differentially expressed at 10°C

or 37°C relative to 22°C in WT, *cca1lhy* or *prr7prr9*, and that were also differentially expressed between WT and clock mutants at similar temperature stresses. Thus, these genes responded to heat (150 genes), cold (55 genes), or both (8 genes) stresses, and are also regulated by the clock (**Supplementary Dataset 1.S6**). As mentioned earlier, the *cca1lhy* and *prr7prr9* double mutants have compromised circadian phasing, it is possible that the observed differential response to temperature stress for many of the 213 genes are a result of phase changes and/or indirect feedback regulation (Farré et al., 2005; Ding et al., 2007; Zhang et al., 2013a).

Therefore, to systematically determine the contribution of the clock mutation in response to heat or moderate cold stress, we filtered the 213 DEGs mentioned above by only considering DEGs having (i) a similar temperature response in both WT and clock mutant *(i.e* up-regulated in response to 37°C compared to 22°C in both WT and mutant for example), (ii) a differential expression between the WT and clock mutant at the specific stress temperature, but (iii) no differential expression between the WT and clock mutant at normal growth temperature (22°C). This led to the identification of 69 DEGs for which the magnitude of the transcriptional response to temperature stress is modulated by the clock (**Supplementary Dataset 1.S6**). These DEGs included genes that are either known to be clock controlled and/or abiotic stress regulated such as *EARLY LIGHT-INDUCABLE PROTEIN (ELIP1; AT3G22840)* and *PHOTOSYSTEM II LIGHT HARVESTING COMPLEX GENE 2.3 (LHCB2.3; AT3G27690)* (**Supplementary Dataset 1.S6**). To determine if these 69 DEGs are direct clock target genes, we compared this list of genes with published CCA1, LHY, PRR7, and PRR9 Chromatin Immunoprecipitation followed by deep sequencing (ChIP-seq) (**Supplementary Dataset 1.S6**) (Liu et al., 2013, 2016; Nagel et al., 2015; Kamioka et al., 2016; Adams et al., 2018). Approximately 25% (17 of 69) of these DEGs were found to be directs targets of one or more clock genes (**Figure 1.4A; Supplementary Figure 1.S3A and Dataset S6**).

Although several of the 17 direct clock targets exhibit circadian oscillations based on the DIURNAL project, a direct link between the clock and their response to temperature stress was previously unknown (Mockler et al., 2007). For example, the plant specific *PLATZ TF (PLATZ2; AT1G76590),* has been reported to have roles in abiotic stress responses such as desiccation tolerance in *Arabidopsis* (Kim et al., 2018). In our dataset, *PLATZ2* is significantly upregulated only at 37ºC in *cca1lhy* (LFC 2.2; FDR 1.3^{E-05}) relative to WT (LFC 1.2; FDR 4.1^{E-03}; **Figure 1.4B and Supplementary Dataset 1.S6**). In addition, *PLATZ2* expression levels is altered in the *cca1lhy* mutant significantly at ZT0, which coincides with the peak expression of *CCA1* and *LHY* though expression changes are also observed at other time-points throughout the day, including ZT8, the timing of peak expression for *PLATZ2* (**Figure 1.4C and Supplementary Figure 1.S3B and 1.S3C**) (Mockler et al., 2007). These results suggest that the expression of *PLATZ2* might be negatively regulated by the clock, and the occurrence of the temperature stress response is also directly controlled by the clock.

CCA1 and LHY modulation of transcript abundance in response to cold stress is also observed for *CYCLING DOF FACTOR 6 (CDF6; AT1G26790)*, a member of the *Dof* subfamily, involved almost exclusively in photoperiod flowering and abiotic stress responses in *Arabidopsis* (**Figure 1.4D**) (Seaton et al., 2015; Krahmer et al., 2018). Similar to other members of the family (*CDF1, CDF2, CDF3* and *CDF5*), *CDF6* shows peak expression in the early morning (**Figure 1.4E**) (Seaton et al., 2015; Krahmer et al., 2018). Some of these *CDFs* are also regulated in the morning by CCA1 and LHY and in the afternoon by PRR9, PRR7, and PRR5 (Niwa et al., 2007; Nakamichi et al., 2012). Consistent with this observation, *CDF6* showed peak expression in the morning that is significantly reduced expression in the *cca1lhy* mutant (**Figure 1.4D and 4E**). Because CCA1 and LHY are suggested to play a positive role on components of the morning loop such as *PRR7* and *PRR9*, the reduced expression of *CDF6* in the *cca1lhy* mutants might be due to

the direct regulation by CCA1 and/or LHY, since *CDF6* is reported to be a target of LHY (Farré et al., 2005; Adams et al., 2018). Alternatively, the reduced expression of *CDF6* in the *cca1lhy* mutant might be due to indirect feedback regulation given that *CDF6* is also a target of PRR9 (Liu et al., 2016). Interestingly, the significant induction of *CDF6* at 10ºC is reduced almost two-fold in the *cca1lhy* mutant, emphasizing the important role of CCA1 and LHY in the cold response of *CDFs* (**Figure 1.4D and 4E**).

For the single DEG that was *mis*-regulated in *prr7prr9/WT* comparison based on our stringent selection criteria, *GALACTINOL SYNTHASE 3 (GolS3; AT1G09350)*, we observed up-regulation in *prr7prr9* relative to WT at 10°C and likely 37°C (**Figure 1.4F**). *GolS3* belongs to a gene family that has been implicated in drought stress, heat stress, cold stress and oxidative damage (Taji et al., 2002). However, very little is known about the precise function of *GolS3*, and the importance of the clock and cold temperature.

Of note, comparison between the time of day heat stress responsive DEGs mentioned in Figure 1.3 and the ChIP-seq datasets, revealed that an additional ~11% (166 genes) were targets of CCA1, LHY, PRR7, or PRR9, 5 of which are shared with the 69 DEGs mentioned above, suggesting that both time of day and the clock modulate their transcriptional response to high temperature stress (**Supplementary Dataset 1.S6**). For example, differential transcriptional response to heat stress for *HSFA3* described in reference to Figure 1.2A above, appears to be modulated by both time of day and the clock (**Figure 1.4A**).

It is possible that the DEGs that were not identified as direct clock targets are indirectly regulated by the clock. In addition, although we were able to make direct connections between the clock and a subset of our DEGs, the ChIP-seq experiments were performed at normal growth temperature for *Arabidopsis* (~22°C). CCA1 has been shown to bind more strongly to *PRR9* at 27ºC compared to 12ºC, and temperature has been implicated in the regulation of alternative

splicing for some clock genes suggesting that both differential binding of clock genes and post-transcriptional regulation might contribute to modulating the gene expression of stress responsive genes (Portolés and Más, 2010; Filichkin et al., 2015). Therefore, ChIP-seq analysis of CCA1, LHY, PRR7, and PRR9 conducted under similar temperature stress conditions used in this study will likely contribute to defining the regulatory relationship between the clock and temperature-responsive genes. In fact, ChIP-seq analysis of evening expressed clock genes (*LUX, ELF3*, and *ELF4*) found that association of these components to target gene promoters was either decreased by high ambient (27°C) or increased at low (17°C) ambient temperatures relative to 22°C, enabling the identification of additional targets that would have otherwise been overlooked (Box et al., 2015; Ezer et al., 2017).

Clock-controlled and Heat Stress Regulated DEGs Reveals Specific Network Connections

Overall, from the DEGs including the time of day specific and clock controlled DEGs obtained in this analysis, 9% are annotated as TFs. From the genes targeted by CCA1, LHY, PRR7, or PRR9, we selected TFs that showed time of day specific differential changes in response to heat and those whose magnitude of the temperature response was controlled by the clock in our analyses. Of these 74 TFs, six are from the 69 clock-controlled DEGs and one belongs to the 119 time of day specific DEGs described above. We examined the Arabidopsis Cistrome Atlas which contains TFs and their target genes obtained by DNA affinity purification sequencing (DAP-seq) (O'Malley et al., 2016), and found targets for one of these seven DEGs, *HB21, HOMEOBOX PROTEIN 21 (AT2G18550)*, a class I *Homeodomain leucine zipper (HD-ZIP)* TF (González-Grandío et al., 2017). *HB21* has been shown to be involved in hormone responses and plant development in light limiting conditions (González-Grandío et al., 2017).

We generated a gene regulatory network composed of 148 TFs including CCA1, LHY, PRR7, PRR9, and HB21 and their respective TF targets that responded to heat in a time of day and/or in a clock dependent context and revealed 208 total connections (**Figure 1.5A and Supplementary Dataset 1.S7**). At all levels (clock regulated or *HB21* regulated DEGs), ~50% of the target genes were either up- or down-regulated in response to heat and based on GO function analysis are enriched for leaf senescence, response to hormones, and response to light stimulus, consistent with the reported function of *HB21* (**Figure 1.5A**). In this hierarchical network, *HB21* targets 97 TFs including 70 that are not targeted by the clock components. Interestingly, the transcriptional response of *HB21* to heat stress is modulated by time of day and showed a significant fold increase in expression at ZT6 in our RNA-seq analysis and confirmed by qRT-PCR (**Figure 1.5B**). In addition, a previously conducted ChIP-seq analysis revealed *HB21* as a direct target of CCA1, however, in our data, *HB21* is not significantly upregulated in response to heat stress (37°C vs 22°C) in the *cca1lhy* or *prr7prr9* mutant or compared to WT (**Supplementary Dataset 1.S1**) (Nagel et al., 2015). Interestingly, 28 HB21 target genes are also targets of CCA1, LHY, PRR7 or PRR9, such as *PLATZ2,* whose response to heat stress is modulated by the clock (**Figure 1.4B, 4C, and 5A**). To the best of our knowledge, this is the first report linking HB21 and ~100 of its target genes to heat stress, time of day specific expression, and the clock in *Arabidopsis*. This integration of multiple data sources allowed us to build a hierarchical regulatory network that highlights the contribution of the dimension of time to specific environmental responses and growth and development. Future work to define the underlying mechanisms of this interaction along with integration of other stressomes will significantly contribute to our understanding on how plants interact with a changing environment.

Conclusion

One of the mechanisms used by plants to respond to environmental stresses is coordinated through an elaborate gene regulatory network that involves the circadian clock. For heat stress responses, understanding how time of day and the circadian clock alters the transcriptional response such as the occurrence and magnitude of the response is essential. In this study, we performed a transcriptomic analysis to assess the extent of the time dimension and the contribution of the clock on the transcriptional dynamics of temperature stress responses during the day when plants should be primed in anticipation of increasing temperatures. Our analysis revealed that although a large subset of genes is induced by heat stress during the early morning and early afternoon, the occurrence or magnitude of this transcriptional induction depends on the time of day and/or the circadian clock. The precise regulatory mechanisms underlying this clock environment interaction in terms of heat stress remains to be determined. Our data reflects responses limited to 1 h and two time-points, therefore future work to detect heat stress responses that accumulate after prolonged exposure or different times of the day will further provide insights to the contribution of time of day on temperature stress responses. DEGs identified in this study should help to guide mechanistic studies and integrate intricate gene regulatory networks together with the massive amounts of publicly available transcriptomic data underlying the abiotic stress response pathways.

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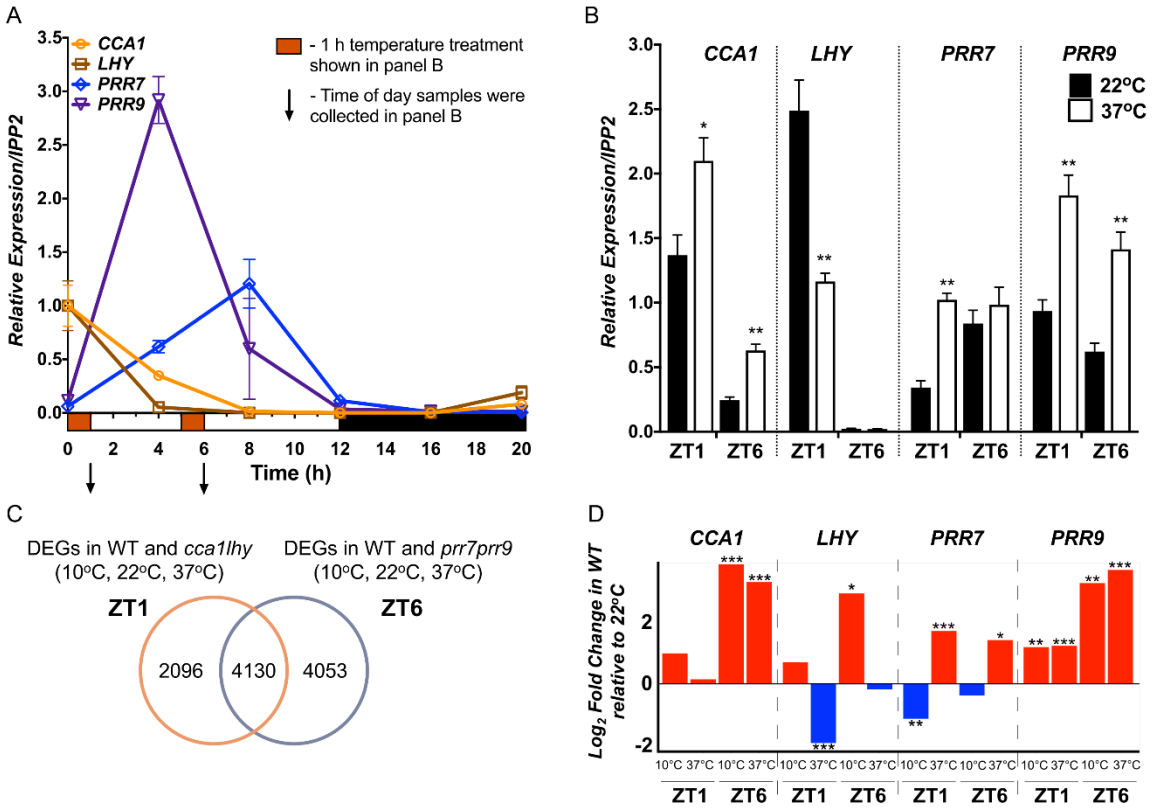
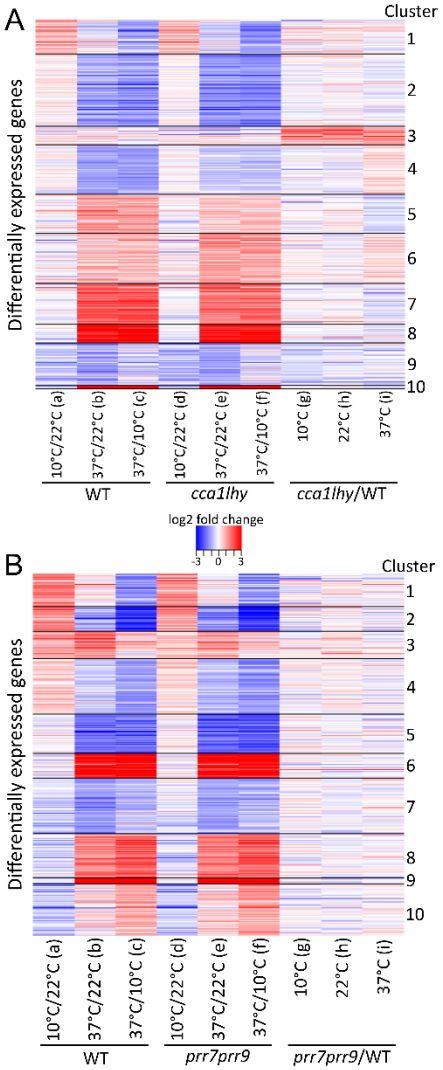
Figures

Figure 1.1: Targeted and global gene expression in response to temperature stress at specific times of the day.



(A) Expression profile of CCA1, LHY, PRR7 and PRR9 in WT plants grown in 12 h Light:12 h Dark (LD) cycles for 12 d by qRT-PCR to demonstrate the time of peak expression. Hashed box indicates when plants were transferred to 10°C or 37°C, and down arrow when samples were collected following temperature treatment, for the results shown in panel B. X-axis, time in hours (h) and Y-axis, relative expression. (B) Changes in transcript abundance of CCA1, LHY, PRR7, and PRR9 following 1 h heat stress treatment (37°C) as indicated in panel A. mRNA levels were normalized to IPP2 and PP2A expression (mean values ± SD, n = 3, three independent experiments). **P ≤ 0.01; *P≤ 0.05, unpaired student t-test. X-axis, time of day samples were collected and Y-axis, relative expression. (C) Venn diagram depicting the overlapping DEGs between the cca1lhy compared to WT at all temperatures (left circle) and the prr7prr9 compared to WT at all temperature (right circle) datasets. (D) Log_2 Fold Change (LFC) for CCA1, LHY, PRR7, and PRR9 in WT at 10°C or 37°C compared to 22°C for ZT1 and ZT6 from our RNA-seq data. ***FDR ≤ 0.001; **FDR ≤ 0.01; *FDR ≤ 0.05.

Figure 1.2: Temperature stress perturb the transcriptome.



C

Cluster	Number of DEGs	Clock genes	Log2 fold change profile	Top 3 enriched biological processes	Fold enrichment
1	566	*PHYA*		transcription factor import into nucleus negative regulation of leaf development negative regulation of leaf senescence	34.4 15.5 15.5
2	1224			thalianol metabolic process S adenosylmethionine metabolic process defense response by callose deposition in cell wall	23.6 10.5 8.8
3	316	*ELF3,ELF4, GI,LUX,PRR3 PRR5,TOC1*		rhythmic process circadian rhythm response to cold	8.7 8.2 4.2
4	831	*LHY*		chloroplast accumulation movement chloroplast avoidance movement establishment of plastid localization	19.7 19.1 17.2
5	662	*CKB3*		response to abscisic acid response to alcohol response to lipid	2.4 2.4 2.2
6	840	*PIF3 PRR9*		nucleic acid templated transcription transcription DNA templated RNA biosynthetic process	1.6 1.6 1.6
7	693	*PRR7*		response to heat response to water deprivation response to water	4.4 4.2 4.1
8	320			heat acclimation chaperone mediated protein folding response to heat	19.0 18.4 12.4
9	721	*CCA1 PHYB*		sulfate reduction ribosomal large subunit assembly sulfate assimilation	29.9 16.6 13.3
10	53			response to hydrogen peroxide response to high light intensity response to heat	136.4 114.3 90.1

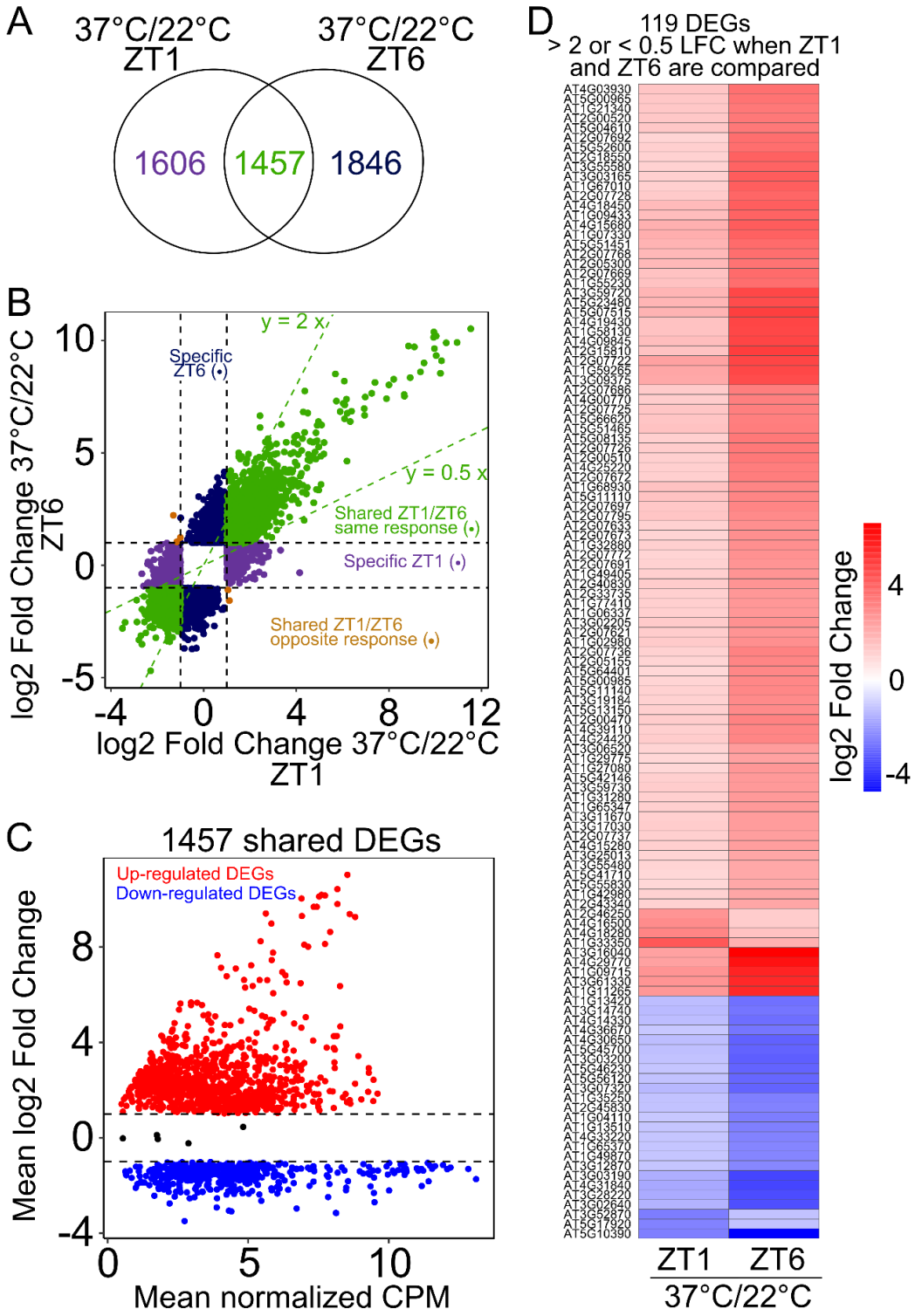
WT *cca1lhy* *cca1lhy/WT* *prr7prr9* *prr7prr9/WT*

D

Cluster	Number of DEGs	Clock genes	Log2 fold change profile	Top 3 enriched biological processes	Fold enrichment
1	716			apocarotenoid catabolic process sesquiterpenoid catabolic process abscisic acid catabolic process	24.1 24.1 24.1
2	545	*LHY*		nicotianamine biosynthetic process nicotianamine metabolic process tricarboxylic acid biosynthetic process	39.4 39.4 39.4
3	593	*CCA1 PRR5, PRR9 ZTL*		circadian rhythm rhythmic process response to water deprivation	5.0 4.6 3.6
4	1231	*PHYB*		aromatic amino acid family biosynthetic process nucleotide-sugar metabolic process nucleoside diphosphate phosphorylation	4.6 4.4 3.9
5	878			nucleosome assembly chromatin assembly nucleosome organization	8.2 6.6 6.4
6	532			heat acclimation response to heat protein folding	13.8 8.0 6.3
7	1227	*ELF4*		photosynthesis, light harvesting in photosystem I ribosomal large subunit assembly ribosomal large subunit biogenesis	9.8 8.3 5.8
8	999	*CKB3 PRR7*		organonitrogen compound biosynthetic process	0.3
9	126			response to hydrogen peroxide response to high light intensity response to heat	70.9 68.2 55.8
10	1336	*CRY2, ELF3 LUX, PHYA PRR3, TOC1*		photoperiodism, flowering regulation of post-embryonic development RNA processing	4.5 2.2 2

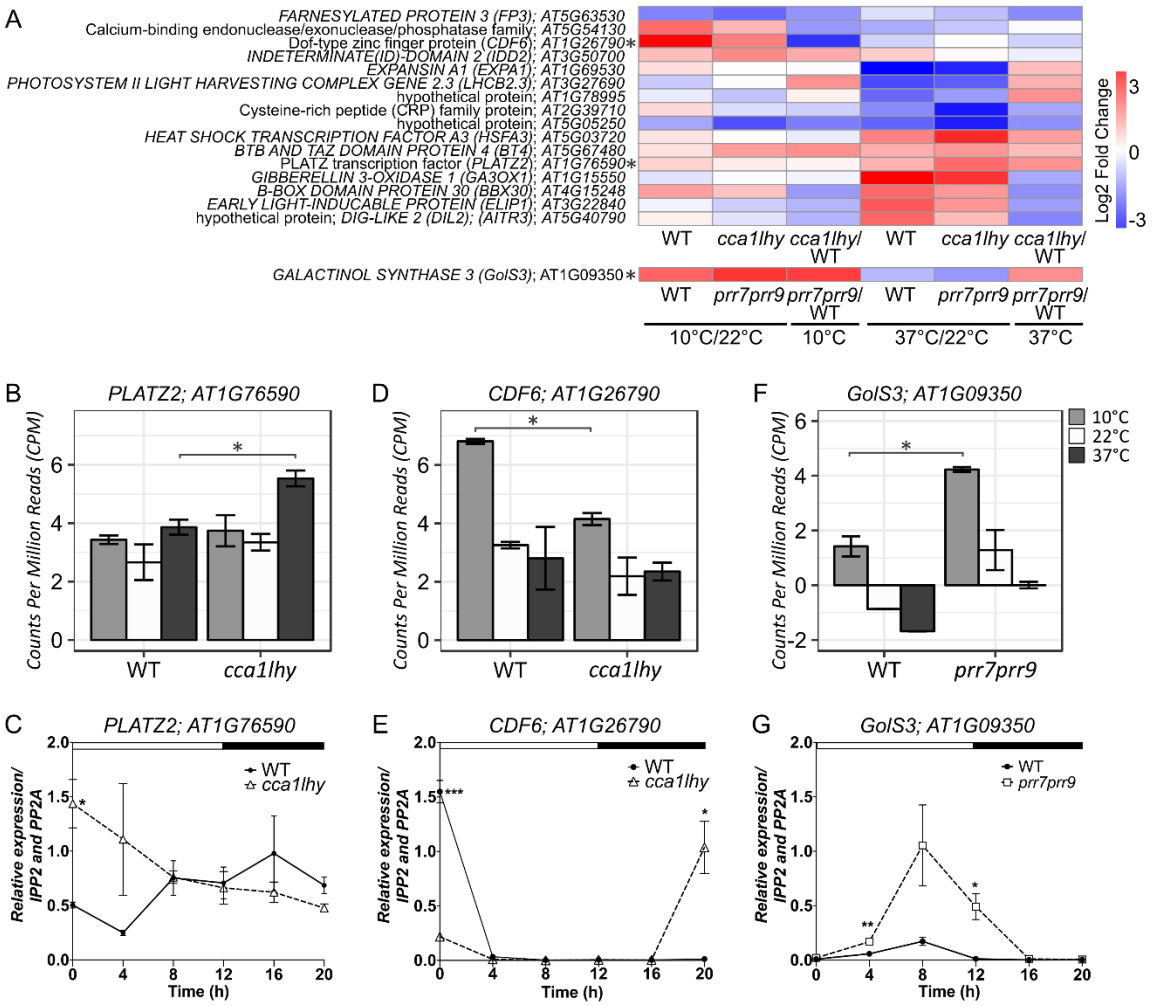
(A and B) Heatmap showing differential expression patterns for DEGs (filtered for LFC > |1| and FDR < 0.05) in *cca1lhy* or *prr7prr9* compared to WT at all temperatures. (C and D) Summary by cluster of number of genes, clock gene location, general expression pattern measured by LFC, and top 3 enriched biological process gene ontology (GO) terms.

Figure 1.3: Time of day modulation of heat stress responses



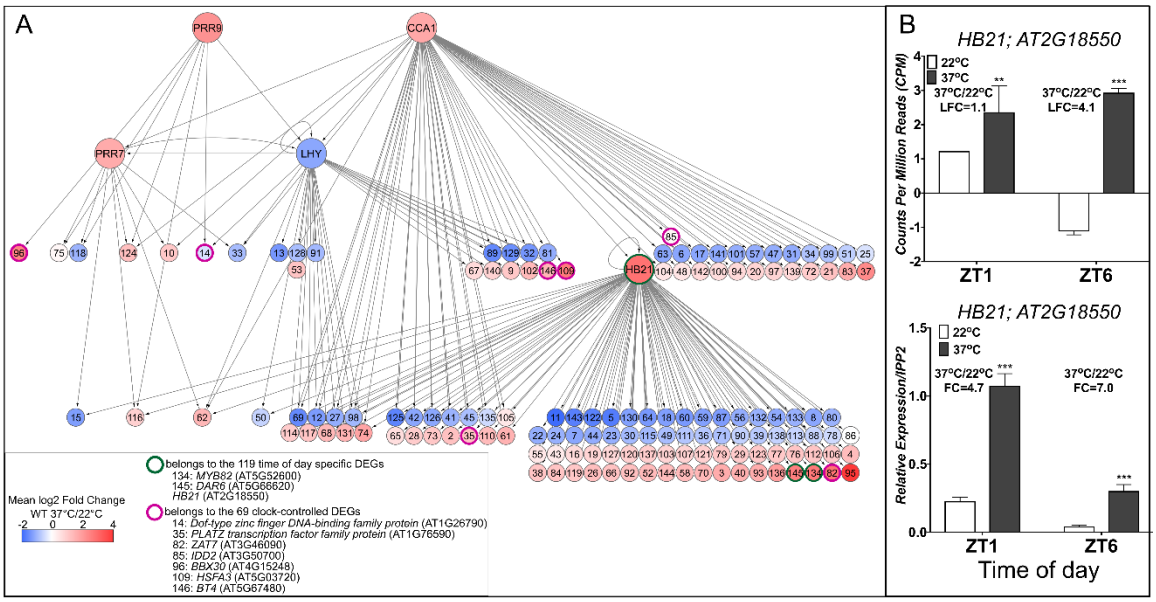
(A) Venn diagram representing overlap of genes differentially expressed at ZT1 and ZT6 in response to 37°C compared to 22°C. (B) Scatter plot comparing Log_2 Fold Change (LFC) values at ZT1 and ZT6 for the genes differentially expressed in response to 37°C compared to 22°C. Genes specific for ZT1 (1606, purple dots), ZT6 (1846, blue dots), and shared between ZT1 and ZT6 (1457, green dots) are plotted. Brown dots represent 5 DEGs that showed opposite expression in ZT1 vs ZT6 (C) Scatter plot representing mean LFC and mean normalized counts per million (CPM) reads for the shared (1457) genes upregulated (red dots) or downregulated (blue dots) in response to 37°C compared to 22°C. Black dots indicated the 5 DEGs with opposite expression. For each gene, mean of the LFC at ZT1 and ZT6 and the normalized CPM at ZT1 and ZT6 for 22°C and 37°C are represented. (D) Heatmap showing LFC values at ZT1 and ZT6 for 119 selected heat responsive DEGs with a LFC either ZT1 or ZT6 with a ratio $y > 2x$ or $y < 0.5x$ when these two timepoints were compared.

Figure 1.4: Alteration of gene expression for select clock controlled targets.



(A) Heatmap of the 17 DEGs that are *mis*-expressed in either *cca1lhy* or *prr7prr9* or relative to WT and are direct ChIP-seq targets of CCA1, LHY, PRR7, or PRR9. Asterisk indicates the 3 genes described in the panels below. (B, D and F) Normalized counts per million (CPM) for *AT1G26790 (CDF6)*, *AT1G76590 (PLATZ2)*, and *AT1G09350 (GolS3)* at 10ºC, 22ºC, or 37ºC. Replicates were averaged and error bars calculated by standard deviation. Significance was determined by LFC > |1| and FDR < 0.05. (C, E, and G) qRT-PCR of *AT1G26790*, *AT1G76590*, and *AT1G09350* transcript levels in WT, *cca1lhy*, and *prr7prr9* plants grown in LD cycles for 12 d. mRNA levels were normalized to *IPP2* and *PP2A* expression (mean values ± SD, n = 3, three independent experiments). ***P≤ 0.0005; **P ≤ 0.005; *P≤ 0.05, unpaired student t-test.

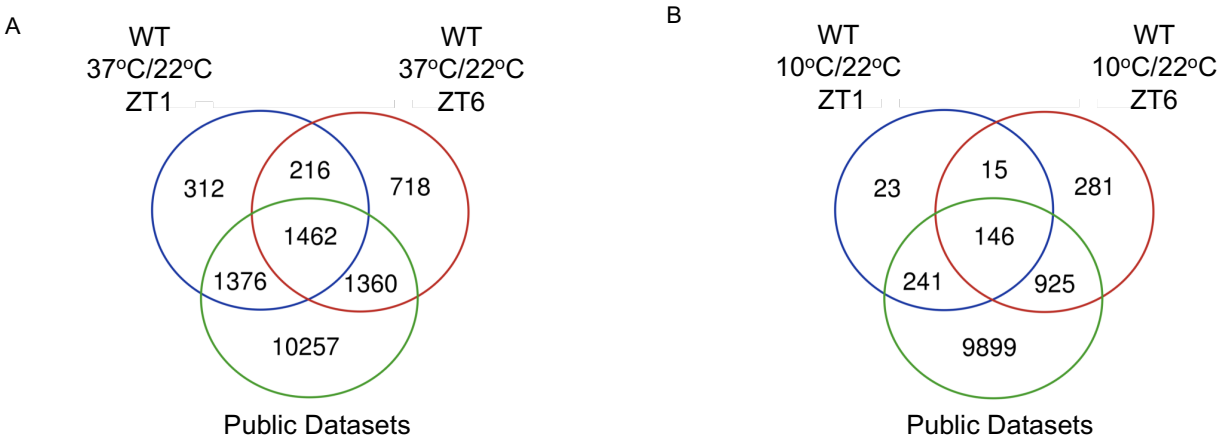
Figure 1.5: Interaction network of differentially expressed transcription factors.



(A) Listed in the first level of the network are the 46 specific TFs including *HB21* that are direct targets of CCA1, LHY, PRR7, and/or PRR9 based on published ChIP-seq datasets (Nagel et al., 2015; Liu et al., 2013; Liu et al., 2016). Second level indicates direct connections for the 97 HB21 targeted DEGs from our dataset, obtained from DNA affinity purification sequencing (DAP-seq) data (O'Malley et al., 2016). The 28 TFs (DEGs) shared between CCA1, LHY, PRR7, PRR9, and HB21 are indicated. Arrows in the network represents an interaction between the TF and its target gene based on either ChIP-seq or DAP-seq. TFs circled in pink are from the list of clock controlled DEGs, and in green are genes from the time of day regulated DEGs. Upregulated and down regulated DEGs in response to 37°C compared to 22°C are indicated by shades of red or blue depending on the LFC values, respectively. Identity of the genes represented by numbers in each connection are listed in Supplementary Dataset 1.S7. Network was visualized using Cytoscape software 3.3.0 (Shannon et al., 2003). (B) Time of day expression of *HB21* based on RNA-seq and qRT-PCR analysis. For RNA-seq, data are based on Normalized Counts Per Million Reads (CPM), and LFC was calculated based on 37ºC/22ºC at either ZT1 or ZT6. qRT-PCR was performed as described in Figure 1.1 and normalized to *IPP2* expression, and Fold Change (FC) was calculated based on expression values at 37ºC relative to 22ºC. ***P≤ 0.0005 and **P ≤ 0.005, unpaired student t-test.

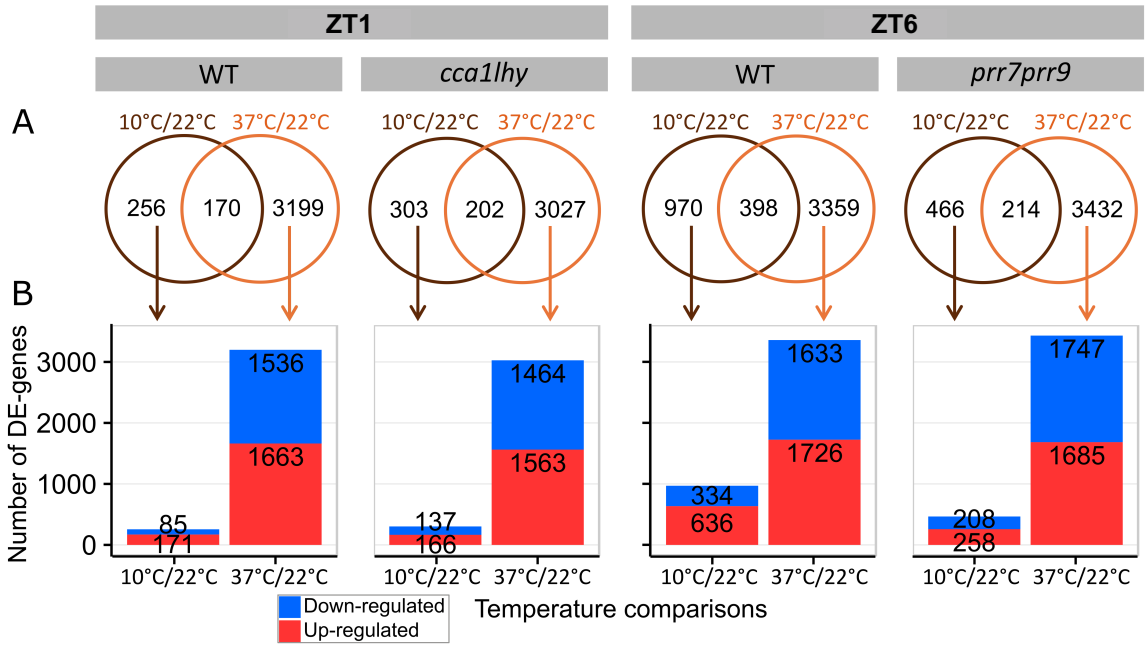
Supplementary Figures and Tables

Supplementary Figure 1.S1: Comparison between our dataset and published datasets.



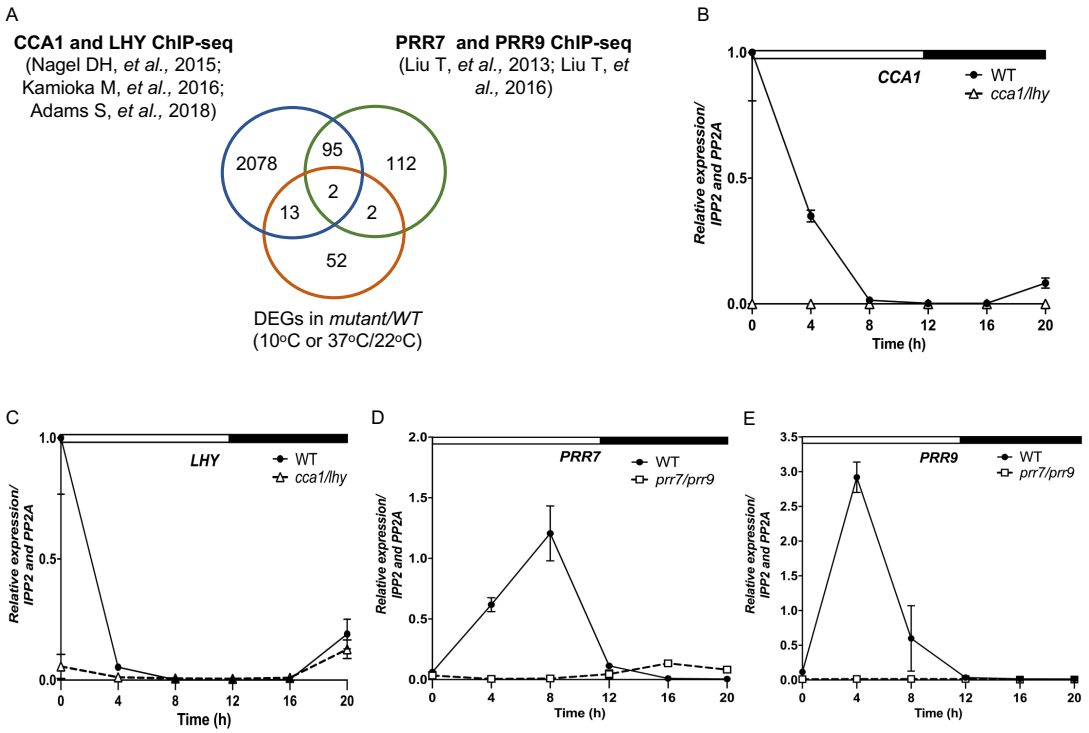
(A and B) Venn diagrams displaying overlapping DEGs between heat (A) and cold (B) stress in WT at ZT1 and ZT6 compared to published datasets available on Genevestigator, and most recent heat and cold stress RNA-seq datasets at the time of this analysis (Calixto et al., 2018; Hruz et al., 2008; Albihlal et al., 2018). For the Calixto et al., 2018 dataset, we used DEGs from timepoints similar to the time of day (ZT0 and ZT6) for our analysis. For the Albihlal et al., 2018, we used RNA-seq from the WT heat stressed, WT non-stressed (NS), and the HSFA1b OX NS experiments.

Supplementary Figure 1.S2: Comparison of the number of up and down-regulated genes at different temperatures.



(A) Venn diagrams display DEGs that overlap between temperature treatments in WT or *cca1lhy* at ZT1 and WT or *prr7prr9* at ZT6 after lights ON. (B) Number of differentially expressed genes comparing temperature treatments by genotype and time of day.

Supplementary Figure 1.S3: Isolation of direct clock targets and the expression profile of the respective clock genes.



(A) Comparison between CCA1, LHY, PRR7, and PRR9 direct targets and differentially regulated genes (DEGs) in *cca1lhy*/WT and *prr7prr9*/WT. Venn diagram showing the overlap between the high confidence CCA1 targets in LD (Nagel *et al.*, 2015), non-overlapping CCA1 targets (Kamioka *et al.*, 2016), LHY targets (Adams *et al.*, 2018), PRR7 targets (Liu *et al.* 2013), and PRR9 targets (Liu *et al.* 2016), and DEGs for *mutants* compared to WT. (B-E) Relative expression of *CCA1, LHY, PRR7,* and *PRR9*. qRT-PCR of *CCA1, LHY, PRR7*, and *PRR9* transcript levels in WT, *cca1lhy*, and *prr7prr9* plants grown in LD after 10 d entrainment in 12 h:12 h LD cycles with collection on 12 d. mRNA levels were normalized to *IPP2* and *PP2A* expression (mean values ± SD, n = 3, three independent experiments). X-axis, time in hours (h) and Y-axis, relative expression.

Supplementary Table 1.S1: PCR Conditions and Primer Sequences used in qRT-PCR

PCR conditions were 95 °C for 3 min followed by 40 cycles at 95 °C for 10 s, 55 °C for 15 s, and 72 °C for 15 s. *Protein Phosphatase 2A (PP2A) and isopentenyl-diphosphate delta-isomerase II (IPP2)* were used as normalization controls.

PRR9-F: 5'-GCCAGAGAGAAGCTGCATTGA-3'

PRR9-R: 5'-CCTGCTCTGGTACCGAACCTT-3'

LHY-F: 5'-CAACAGCAACAACAATGCAACTAC-3'

LHY-R: 5'-AGAGAGCCTGAAACGCTATACG-3'

PRR7-F: 5'-CTTTCTCAAGGTATAATCCAGCC-3'

PRR7-R: 5'-ACAATCATATGCTGCTTCAGTC-3'

CCA1-F: 5'-CAGCTCCAATATAACCGATCCAT-3'

CCA1-R: 5'-CAATTCGACCCTCGTCAGACA-3'

GolS3-AT1G09350-F: 5'-ACAGGCCAAGAAGGAAATATGG-3'

GolS3-AT1G09350-R: 5'-GATGGAGCTTTGGCACATTG-3'

PLATZ2-AT1G76590-F: 5'-TGGCTAATCCCAATGCTAAGAG-3'

PLATZ2-AT1G76590-R: 5'-ACATGTTGCACTCGCTTTTG-3'

CDF6-AT1G26790-F: 5'-GACTTGTATTGTCAGTAACAGATTGG-3'

CDF6-AT1G26790-R: 5'-TGGCTGGACAATTACACCG-3'

HB21-AT2G18550-F: 5'-CTTCTACTCATTTCTCAATTGTACCC-3'

HB21-AT2G18550-R: 5'- CACCCATTGCCTTCGTTTTC-3'

IPP2-F: 5'-GTATGAGTTGCTTCTGGAGCAAAG-3'

IPP2-R: 5'-GAGGATGGCTGCAACAAGTGT-3'

PP2A-F: 5'-TAACGTGGCCAAAATGATGC-3'

PP2A-F: 5'-GTTCTCCACAACCGATTGGT-3'

List of Supplementary Datasets/Files

Supplementary Dataset 1.S1: List of all *Arabidopsis* and differentially expressed genes

Supplementary Dataset 1.S2: List of all differentially expressed genes represented in the venn diagrams

Supplementary Dataset 1.S3: Significantly up and down-regulated genes in clock mutants and wild-type by cluster represented in the heatmaps

Supplementary Dataset 1.S4: Gene Ontology (GO) terms enriched in differentially expressed genes

Supplementary Dataset 1.S5: Time of day regulated DEGs

Supplementary Dataset 1.S6: Clock regulated DEGs

Supplementary Dataset 1.S7: Network interactions between differentially expressed TFs

Chapter 2

Clock controlled and cold-induced *CYCLING DOF FACTOR6* alters growth and development in Arabidopsis

Abstract

The circadian clock represents a critical regulatory network, which allows plants to anticipate environmental changes as inputs and promote plant survival by regulating various physiological outputs. Here, we examine the function of the clock-regulated transcription factor, CYCLING DOF FACTOR 6 (CDF6), during cold stress in *Arabidopsis thaliana*. We found that the clock gates *CDF6* transcript accumulation in the vasculature during cold stress. *CDF6* mis-expression results in an altered flowering phenotype during both ambient and cold stress. A genome-wide transcriptome analysis links CDF6 to genes associated with flowering and seed germination during cold and ambient temperatures, respectively. Analysis of key floral regulators indicates that CDF6 alters flowering during cold stress by repressing photoperiodic flowering components, *FLOWERING LOCUS T* (*FT*), *CONSTANS* (*CO*), and *BROTHER OF FT (BFT)*. Gene ontology enrichment further suggests that *CDF6* regulates circadian and developmental associated genes. These results provide insights into how the clock-controlled CDF6 modulates plant development during moderate cold stress.

Introduction

The circadian clock consists of an expansive regulatory network, which enables eukaryotic organisms to synchronize their metabolism, physiology, and development to daily and seasonal environmental changes (Greenham and McClung, 2015; Creux and Harmer, 2019). Through a coordinated and interconnected series of transcriptional-translational feedback regulation between multiple components, the clock modulates the expression of a large proportion of the transcriptome in plants. For example, the clock regulates 40-50% of genes involved in plant abiotic stress responses (Covington et al., 2008). Several recent transcriptome studies indicate that the time of day impacts the plant transcriptional response to abiotic stimulus (Wilkins et al., 2010; Blair et al., 2019; Grinevich et al., 2019; Bonnot et al., 2021; Markham and Greenham, 2021). The clock is also involved in regulating several critical developmental phenotypes. For example, mis-expression of some clock components (*CIRCADIAN CLOCK 1/CCA1, EARLY FLOWERING 3/ELF3, PSEUDO RESPONSE REGULATOR 9/PRR9, TIMING OF CAB EXPRESSION 1/TOC1, etc.*) results in altered hypocotyl growth and flowering (Nagel and Kay, 2012; Huang and Nusinow, 2016; Nakamichi, 2020).

In Arabidopsis, the clock coordinates aspects of photoperiodic flowering primarily through regulation of GIGANTEA (GI) (Song et al., 2015). During long-day (16 h light: 8 h dark) conditions, GI forms a complex with FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) to target degradation of *CYCLING DOF FACTORS* (*CDFs*), a small subfamily of the DNA-binding with one finger (DOF) transcription factor (TF) family, which enables *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) to accumulate and promote flowering (Imaizumi et al., 2005; Sawa et al., 2007; Fornara et al., 2009; Nohales et al., 2019). However, during short-day conditions, the CDFs bind to the DOF binding sites (AAAG) of *FT* and *CO* and act redundantly to suppress the accumulation of *FT* and *CO* mRNAs, resulting in delayed flowering (Imaizumi et al., 2005; Fornara

et al., 2009). Recent work indicates that the vasculature-expressed *CDF6*, negatively regulates *FT* transcript abundance, resulting in delayed flowering (Krahmer et al., 2019). CDF6 also interacts with GI; consistent with the known mechanism for the GI-FKF1 module degrading CDF family members during long-day conditions (Krahmer et al., 2019).

The *CDFs* are regulated by several clock components. For example, the day-expressed PRR9, PRR7, and PRR5 sequentially bind to the *CDF5* promoter region to repress its accumulation during the day (Martín et al., 2018). PRR7 and PRR5 associate with the promoter region of *CDF3* (Nakamichi et al., 2012). Furthermore, PRR9, PRR7, and PRR5 negatively regulate the expression of *CDF1,* while CCA1 positively regulates the *CDF1* expression via regulation of *GI* (Nakamichi et al., 2007). Expression of both *CDF5* and its natural antisense transcript, *CDF5 LONG NONCODING RNA (FLORE)* are altered in *CCA1* over-expression lines (Henriques et al., 2017). In addition, the expression of the lesser characterized *CDF6* is also clock-regulated, and PRR9 and LHY (LATE ELONGATED HYPOCOTYL) directly bind to the *CDF6* promoter (Liu et al., 2016; Adams et al., 2018; Blair et al., 2019).

Photoperiodic flowering regulators are involved in various abiotic stress responses. GI is required for the drought escape, oxidative stress, and cold stress response (Riboni et al., 2013; Fornara et al., 2015). CDF3 confers tolerance to drought and freezing temperatures, while acting in both GI-dependent and GI-independent abiotic stress response pathways (Corrales et al., 2017; Renau-Morata et al., 2020). Furthermore, plants over-expressing the tomato orthologs of *CDF1* and *CDF3* show increased tolerance during drought and salinity stress (Corrales et al., 2014). *CDF1, 2, 3, 5,* and *6* are uniformly upregulated in response to cold stress (Kilian et al., 2007; Calixto et al., 2018; Blair et al., 2019). As such, the CDFs are considered part of the cold-regulated (*COR*) genes, due to their altered expression in a transcriptome analysis of the central cold regulators, *C-REPEAT BINDING FACTORS (CBFs)* triple loss-of-function mutants *(cbf123) (Song*

et al. 2021; Shi et al. 2017). Interestingly, *CDF5* and *CDF6* were both induced over two-fold in response to low temperature in parallel with CBF1, CBF2, and CBF3 (Park et al., 2015). While *CDF3* confers cold stress tolerance and *CDF5* impacts hypocotyl elongation during short days (Corrales et al., 2017; Martín et al., 2020), the *CDFs* have not been shown to have differential function in regulating plant growth and development during cold stress.

Here we use a combination of meta-data, transcriptomic, genetic, and phenotypic approaches to better understand the interplay between the clock regulation and the function of the lesser characterized family member, CDF6, during cold stress. We find that CCA1 modulates gating of *CDF6* transcript accumulation in response to moderate cold stress. Through analysis of tissue-specific (*SUC2*) and mutant genotypes, we find that *CDF6* influences photoperiodic flowering in both ambient and cold temperatures, and germination during ambient temperature. Specifically, vasculature-expressed *CDF6* represses *FT, CO,* and *BFT* to regulate flowering. Finally, we show that *CDF6* significantly alters the transcriptome during cold stress including the expression of genes involved in flowering, rhythmic, and metabolic processes.

Materials and Methods

Plant Materials and Growth Conditions

Arabidopsis thaliana seeds were sterilized for 3 - 4 h (~100mL 6% sodium hypochlorite) and ~4mL concentrated hydrochloric acid), plated on 1X Murashige and Skoog (MS) medium supplemented with 1.5% sucrose (w/v) and stratified in the dark for 3 nights at 4°C. Seeds were grown at constant 22°C with ~90 µmol photons$\cdot s^{-1} \cdot m^{-2}$, in diurnal (12 h light:12 h dark; LD) cycles for 8 days. For circadian and time of day experiments, seedlings were transferred to constant light (LL) for 2 days before sampling. Columbia-0 (Col-0) was used as the wild-type (WT) control. Clock genotypes

(*cca1-1/lhy-21*, *CCA1-OX (35S::CCA1)*, *SUC2::CCA1 #18*, *prr7-3/prr9-1*) and *CDF* mis-expressed line (*SUC2::HA-CDF6 #11*) were previously characterized (Farré et al., 2005; Pruneda-Paz et al., 2009; Endo et al., 2014; Nagel et al., 2014; Krahmer et al., 2019). The *cdf12356* quintuple line was generated using the CRISPR/Cas9-based gene editing constructs in pKIR1.1 plasmids (Tsutsui and Higashiyama, 2017) containing *CDF1* (5'- GTTTGGCTGGACAATTACAC-3') and *CDF6* (5'-GTCTCAAGTTAGAGATACTC-3') gRNAs using the *cdf235* triple T-DNA mutants as a genetic background (Fornara et al., 2009). To generate *cdf1* and *cdf6* mutations, the *cdf1235* quadruple mutant was generated first, and then the *cdf6* mutation was induced by gene editing in the *cdf1235* quadruple to generate the *cdf12356* quintuple mutant (**Supplementary Figure 2.S1**). The *cdf6* single mutant is a SALK T-DNA insertion mutant line (SALK_010734), which was genotyped to confirm homozygosity. The reduced *CDF6* expression level was validated via quantitative real-time PCR (qRT-PCR). To generate *pCDF6::CDF6-GUS* plants, the genomic copy with ~1.2 kb of the promoter and the coding sequence was PCR purified and cloned into pENTR d-TOPO (Invitrogen). LR Clonase II was used to perform the Gateway reaction with pMDC162 to create *pCDF6::CDF6-GUS.* The sequences were confirmed via Sanger sequencing (Institute for Integrated Genome Biology (IIGB) Genomics Core, University of California, Riverside (UCR)), and the vectors were transformed into WT plants with *Agrobacterium*-mediated transformation. Two independent T3 lines (Line A and B) were selected for GUS assays. For cold treatment, seedlings were exposed for 1 h to 10°C and sampled at 4 h intervals (ZT12 - ZT36) along with control samples grown at ambient temperature (22°C) after 8 day entrainment in LD and two days of continuous light (LL). For the continuous cold experiments, plants were grown in long-day conditions (16 h light: 8 h dark) for 8 days at 22°C; then seedlings were maintained at 22°C or transferred to 10°C long-day for two days before sampling every 4 h.

Quantitative Real-time PCR

Seedlings were prepared and grown as described above. Total mRNA was isolated with the GeneJET Plant RNA Purification Kits (Thermo Fisher Scientific). cDNA was synthesized using 1 µg of total RNA and was reverse transcribed with the iScript cDNA synthesis kit (Bio-rad). qRT-PCR was performed with SYBR Green Master Mix (Thermo Fisher Scientific) with the CFX384 Touch Real-Time PCR Detection System (Bio-Rad). Three biological and three technical replicates were analyzed. Relative expression was calculated against the housekeeping gene, *ISPOENTENYL-DIPOHSPHATE DELTA ISOMERASE II* (*IPP2*), using the ΔΔCq method. See Supplementary Table 2.S1 for gene specific primer sequences and qRT-PCR conditions used here.

GUS Assay

For Beta-glucuronidase **(**GUS**)** assays plants were grown in long-days (16 h light: 8 h dark), similar to the flowering time experiments below and GUS staining was performed as previously described with the following modifications (Yang et al., 2018). Four seedlings were harvested in 1 mL of cold 90% (v/v) acetone and then vacuumed for 10 min. Seedlings were fixed at room temperature for 60 min. Acetone was replaced with ~500 µl of wash buffer (10 mM EDTA, 50 mM Phosphate buffer (pH 7), 0.1% (v/v) Triton X-100, 1 mM Potassium ferrocyanide, 1 mM Potassium ferricyanide, in 20% Methanol) on ice. After vacuuming three times for 10 min, wash buffer was replaced with ~500 µl of staining buffer (10 mM EDTA, 50 mM Phosphate buffer (pH 7), 0.1% (v/v) Triton X-100, 1 mM Potassium ferrocyanide, 1 mM Potassium ferricyanide, 20% Methanol, 2 mM X-Gluc (Carbosynth Ltd, United Kingdom)). Seeds were vacuum-infiltrated for 40 min and then incubated for 20 min for *pCDF6::CDF6-GUS* line A and ~16 h for line B at 37°C. After

incubation, seedlings were washed and stored in 70% (v/v) ethanol until imaging on a Leica M165 FC stereoscope (Leica Microsystems CMS GmbH, Wetzlar, Germany) using white light.

Flowering Time and Seed Germination Assays

For flowering time assays, seeds were stratified for 2-3 nights after sterilization and grown for ~10 days on 1X MS plates at ~90 μmol photons·s^{-1}·m^{-2}. Seedlings were transferred to soil and grown in long-days (16 h light: 8 h dark) or short days (8 h light: 16 h dark) at 22°C or 10°C as indicated in figure legends. Flowering time was measured as the mean number of rosette leaves for at least three independent replicates with n≥6 plants per genotype. Germination was defined as emergence of the radicle and measured for three biological replicates of 75-100 seeds from WT, *cdf6*, *cdf12356*, and *SUC2::CDF6*, using a modification of Nelson et al., 2009. Briefly, seeds were liquid sterilized with 70% (v/v) ethanol and 0.05% (v/v) Triton X-100 followed by incubations with 70% (v/v) and 95% (v/v) ethanol. Seeds were sprinkled on filter papers on 0.8% (w/v) Bacto-agar plates. After plating, seeds were immediately transferred to constant light (μmol photons·s^{-1}·m^{-2}) at constant 22°C or 10°C.

RNA-sequencing Set-up and Analysis

Three biological replicates of WT, *cdf6*, and *SUC2::CDF6* were grown as described above and sampled at lights on (Zeitgeber Time 0, ZT0) after 1 h 10°C temperature treatment along with control seedlings grown at 22°C. 1 μg of total RNA was isolated and DNAse I treated (Millipore Sigma). mRNA purification and libraries were prepared as described previously (Blair et al., 2019). Final libraries were purified by Ampure XP beads. Library quality was confirmed via Qubit 2.0

Fluorescence Reader (ThermoFisher Scientific) and Bioanalyzer 2100 (Agilent Genomics). The analysis pipeline was also described previously (Blair et al., 2019). Briefly, the sequencing was performed at the UCR IIGB Genomics Core facility on the NextSeq500 (Illumina), which generated single-end 75 bp sequences. Reads were mapped to the TAIR10 genome using Hisat2, and limma.voom was used to determine differential gene expression (Law et al., 2014; Kim et al., 2015; H Backman and Girke, 2016). Differentially expressed genes (DEGs) were defined as genes with $-1 > \text{Log}_2\text{Fold Change} > 1$ and False Discovery Rate < 0.05. Gene Ontology (GO) terms were assigned with a previously published pipeline and flowering-related genes were identified using a previously published dataset (Bonnot et al., 2019; Kinoshita and Richter, 2020).

Meta-data Analysis

The transcription factor binding site motif analysis was conducted by inputting the 500 bp upstream of the DOF-TF family members, from the TAIR bulk data download, into Find Individual Motif Occurrences (FIMO) (Grant et al., 2011; Le Hir and Bellini, 2013). Timing of peak expression (phase) was determined with the Phaser tool from the DIURNAL database (Mockler et al., 2007). The list of *COR* genes was obtained from an analysis of the *cbf1, 2,* and *3* transcriptomes (Shi et al., 2017). The authors used Cuffdiff default parameters to normalize, perform statistical analysis, and identify differentially expressed genes from FPKM values; please refer to the methods section of Shi et al., 2017 for additional details. Upstream clock regulators of DOF TFs were compiled from published Chromatin Immunoprecipitation-sequencing (ChIP-seq) datasets (Huang et al., 2012; Liu et al., 2013, 2016; Nagel et al., 2015; Kamioka et al., 2016; Adams et al., 2018). TFs that may bind to *CDF6 in vitro* were identified using the DNA Affinity Purification (DAP)-sequencing

genome browser by selecting TF families, searching for AT1G26790 (*CDF6*) in the viewer, and checking for peaks that might indicate TF occupancy (O'Malley et al., 2016).

Identifiers of Genes Referenced in this Study

AT5G62430/CDF1, AT5G39660/CDF2, AT3G47500/CDF3, AT2G34140/CDF4, AT1G69570/CDF5, AT1G26790/CDF6, AT1G69572/FLORE, AT2G31230/ERF15, AT1G60960/ATIRT3, AT2G46790/PRR9, AT5G02810/PRR7, AT1G01060/LHY, AT2G46830/CCA1, AT5G15840/CO, AT1G65480/FT, AT1G07887, AT1G53480/MRD1, AT1G53490/HEI10, AT1G76960, AT5G35935, AT5G35940, AT1G60960/ATIRT3, AT1G75945, AT2G06995, AT3G59930, AT5G33355, AT1G68050/FKF1, AT1G68840/ATRAV2, AT1G80340/GA3OX2, AT2G45660/SOC1, AT5G24470/PRR5, AT5G60910/AGL8, AT5G62040/BFT

Data Availability

The sequences reported in this paper have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database and can be accessed through GEO Series accession GSE197581.

Results

CCA1 gates *CDF6* expression in response to cold stress

We previously showed that the expression of *CDF6* is altered in the *cca1lhy* clock mutants under diurnal (12h light: 12h dark; LD) conditions (Blair et al., 2019). In this study we found that

in constant light and temperature, *CDF6* expression is also altered in the *CCA1* over-expressor (*35S:CCA1*), *cca1lhy*, and *prr7prr9* mutants evident by dampened transcript accumulation and changes in peak expression (**Supplementary Figure 2.S2A-C**). Similar to the other CDFs, we observed that *CDF6* is localized to the vasculature (**Figure 2.1A**). We therefore assessed the expression of *CDF6* in previously published lines where *CCA1* is driven by the phloem companion cells (PCC)-specific promoter of *SUCROSE TRANSPORTER 2* (*SUC2::CCA1*), (Endo et al., 2014). Interestingly, when *CCA1* is expressed in the PCC, *CDF6* transcript accumulation is significantly altered relative to WT (Col-0) **(Figure 2.1B)** and to higher extent compared to changes in *35S::CCA1*, specifically between ZT20 and ZT24 **(Supplementary Figure 2.S2A)**, the timepoints where both *CDF6* and *CCA1* transcript level are at the highest in the WT (**Figure 2.1B, Supplementary Figure 2.S3A**). These results indicate that *CCA1* regulates *CDF6* transcript accumulation in the PCC of the vasculature.

A subset of DOF-TFs (*CDF1,2,3,5,6*, *ADOF1*) are *COR* genes based on transcriptomic analysis of *cbf* mutants (Shi et al., 2017). In response to moderate cold stress (10ºC), *CDFs* are mostly upregulated during the morning (Blair et al., 2019**)**. *CDF6* is significantly upregulated at ZT1 but not at ZT6, while *CDF1* and *CDF3* are significantly upregulated at both times of day (Blair et al., 2019**)**. To determine whether CCA1 is responsible for *CDF6* mRNA accumulation during cold stress, we exposed WT, *35S::CCA1, SUC2::CCA1 and cca1lhy* seedlings to 10ºC for 1 h before sampling every 4 h (ZT12 - ZT36) and quantifying *CDF6* transcript abundance. Seedlings were entrained for 8 days in LD before transfer to continuous light (LL). Of note, both *CCA1* and its partially redundant partner, *LHY,* display increased transcript abundance in response to cold depending on time of day (**Supplementary Figure 2.S3A-D**). In WT, we found that *CDF6* transcript abundance, similar to *CCA1*, is significantly elevated in response to cold at the time points surrounding dawn (ZT20 and ZT28) (**Figure 2.1C-2.1D, Supplementary Figure 2.S3A**).

However, *CDF6* displays higher transcript abundance in response to the cold at dawn (ZT24), late morning (ZT28) and dusk (ZT12 and ZT16) in the *35S::CCA1* line (**Figure 2.1E**). While in the *SUC2::CCA1* line, we observed that *CDF6* exhibits higher transcript accumulation across the night period (ZT12 - ZT20) but lower at dawn (ZT24) in response to cold (**Figure 2.1F**). Furthermore, CDF6 induction in response to cold at ZT24 in WT (though not significant) and *35S::CCA1* is significantly reduced in *SUC2::CCA1* but restored in the *cca1lhy* mutants (**Figure 2.1D-2.1F; Supplementary Figure 2.S2D**). However, the induction of *CDF6* transcript abundance at ZT28 in WT, *35S::CCA1*, and *cca1lhy* is abolished in *SUC2::CCA1* (**Figure 2.1D-2.1F; Supplementary Figure 2.S2D**). A comparison between WT, *35::CCA1* and *SUC2::CCA1* further supports the temporal misregulation of *CDF6* in response to cold (**Supplementary Figure 2.S2E**). Similar to what was observed at 22ºC, *CDF6::CDF6-GUS* lines also show vasculature protein accumulation at ZT0 after 1 h exposure to 10ºC (**Supplementary Figure 2.S3E**). Together, these data suggest that the clock via CCA1 gates *CDF6* expression which in turn diminishes the normal cold induction of CDF6 during the day and may promote cold induction during the subjective night.

CDF6 regulates flowering and seed germination

To date, higher orders of multiple mutants of *cdf1*, *cdf2, cdf3*, and *cdf5* are known to cause early flowering, and a more recent study demonstrated that *SUC2::CDF6* results in delayed flowering (Fornara et al., 2009; Krahmer et al., 2019). However, how cold stress impacts CDFs regulation of flowering time is still an open question. To examine further the role of *CDF6* in photoperiodic flowering during cold stress, we measured the flowering phenotype of *SUC2::CDF6* and a SALK T-DNA insertion line for *cdf6* (**Supplementary Figure 2.S4A and 2.S4B**), under continuous ambient (22ºC) or cold (10ºC) temperatures. As the CDFs share high sequence homology and likely

redundant function, we also measured flowering in the *cdf* quintuple mutant (*cdf12356,* **Supplementary Figure 2.S1).** In long-day (16 h light: 8 h dark) conditions, the *SUC2::CDF6* plants displayed late flowering relative to WT under both ambient and cold temperatures (**Figure 2.2A**). Interestingly, the *cdf12356* mutant exhibits significant early flowering under both ambient and cold temperature; however, this observed early flowering phenotype is notably reduced during cold (**Figure 2.2A**). While the *cdf6* single mutant did not show a significant flowering phenotype relative to WT at ambient temperature, under cold temperature, *cdf6* plants display modest but significant early flowering phenotype (**Figure 2.2A**). Furthermore, we observe statistically significant interaction between temperature and genotype (**Supplementary Figure 2.S4C**). These observations suggest that CDF6 plays a redundant role in the regulation of photoperiodic flowering during ambient temperature and a predominant role during cold temperature. In short-day conditions (8 h light: 16 h dark), only *cdf12356* shows a difference relative to WT with early flowering in both cold and ambient temperatures (**Supplementary Figure 2.S4D**).

CDF4 and other DOF-TFs, such as *DOF AFFECTING GERMINATION1* (*DAG1*), *DAG2*, and *DOF6*, have been shown to play a role in germination (Ruta et al., 2020). To determine whether *CDF6* functions similarly to CDF4 and these other DOFs, we measured germination over time in constant light and temperature (22ºC or 10ºC) and in the *SUC2::CDF6, cdf6*, and *cdf12356* lines. *SUC2::CDF6* and *cdf12356* seeds have delayed germination relative to WT during ambient temperature but not under cold, whereas there is no significant difference in *cdf6* germination at either temperature (**Figure 2.2B**). Next, we measured seed dormancy after cold (4ºC) and dark treatment of seeds for 3 nights and found germination synchrony across all genotypes tested (**Supplementary Figure 2.S3E, 2.S3F**). Thus, we conclude that during ambient temperature *CDF6* may contribute to the regulation of seed germination rather than dormancy.

Vasculature-expressed *CDF6* regulates the transcriptome during cold stress

To determine a broader role for *CDF6* in both development and cold responses, we examined transcriptome changes in WT and *CDF6* mis-expression lines (*SUC2::CDF6* and *cdf6*). For this, seedlings were entrained for 8 days in LD and then transferred to constant light for 2 days. On day 11, seedlings were subjected to 10ºC for 1 h and sampled at subjective dawn (ZT0), the time of day *CDF6* expression is significantly altered in the *cdf6* mutant and *SUC2::CDF6* lines (**Supplementary Figure 2.S4B and Supplementary Figure 2.S5F, 2.S5G**). Using a cut-off of -1 > Log_2 Fold Change > 1 and False Discovery Rate (FDR) < 0.05, we identified 473 differentially expressed genes (DEGs) in the WT (10ºC vs 22ºC), and we consider these as the generally cold-responsive DEGs in this study (**Figure 2.3A, Supplementary Dataset 2.S1**). Of these generally cold-responsive genes, ~31% were previously identified as *COR* genes in the *cbf1, 2,* and *3* mutants (**Supplementary Dataset 2.S2**; Shi et al., 2017). Using the Phaser database which provides insight into clock regulation of transcript levels, we identified that ~50% of these DEGs exhibit rhythmic expression with significant enrichment from the afternoon to early evening period; this is consistent with the peak expression for cold-responsive genes and the proportion of the transcriptome that cycles during cold stress (Covington et al., 2008; Grundy et al., 2015; **Supplementary Figure 2.S5A, Supplementary Dataset 2.S3**).

Next, we identified 344 genes that are differentially expressed in *cdf6* at 10ºC compared to 22ºC; ~71% of these are also found in the WT (10ºC vs 22ºC) dataset (**Figure 2.3A, Supplementary Figure 2.S5B**). However, the effect of *SUC2::CDF6* at 10ºC compared to 22ºC is most striking with 1339 DEGs being detected, and of these only ~27% overlapped with the generally cold-responsive genes (**Figure 2.3A, Supplemental Figure 2.S5B**). We speculate that the other ~73% of DEGs may be specifically regulated by *CDF6* in the vasculature in response to cold stress rather than across cell types in response to cold. A Gene Ontology (GO) analysis reveals

an enrichment for terms associated with the clock (Cluster 2), responses to abiotic and biotic factors (Cluster 1, 3, 4, 5), and metabolic processes (Cluster 4, 5) (**Figure 2.3B**). Cluster 2 also contains a number of flowering- and clock-related genes (**Figure 2.3B**).

To further dissect the impact of *CDF6* mis-expression on the transcriptome during cold stress, we compared each genotype relative to WT at each temperature. First, we observed that there are very few DEGs in *cdf6* compared to the WT (16 DEGs at 22ºC; 12 DEGs at 10ºC (**Figure 2.3C**). However, in vasculature-expressed *CDF6* (*SUC2:CDF6*), we observe a greater impact on the transcriptome during cold stress (524 DEGs) compared to ambient (18 DEGs) temperature (**Figure 2.3C**). Cluster 6 genes generally show downregulation in *SUC2::CDF6* compared to WT at 10ºC with minimal expression change in the other comparisons. These genes were enriched for the biological GO term "positive regulation of flowering" and many genes involved with flowering are found in this cluster including the florigen molecule, *FT* (**Figure 2.3B**). The other clusters show enrichment for terms associated with responses to stimulus/stress, immune system processes, and development (**Figure 2.3B**).

We also identified genes that may contribute to the delayed germination phenotype that we previously observed in *SUC2::CDF6* lines under ambient temperatures (**Figure 2.2B, Supplementary Figure 2.S5C**). For example, IRON REGULATED TRANSPORTER 3 (IRT3) transports zinc, which is essential for development of reproductive organs, and is needed for proper seed development (Lee et al., 2021). *IRT3* is upregulated in *SUC2::CDF6* at both temperatures although that upregulation is reduced at 10ºC as compared to 22ºC, suggesting that *SUC2::CDF6* seeds may have increased zinc transport to support germination during ambient temperature (**Supplementary Figure 2.S5C**). In addition, *ETHYLENE RESPONSE FACTOR15* (*ERF15*), a positive regulator of ABA, is upregulated in *SUC2::CDF6* under both ambient and cold temperatures (**Supplementary Figure 2.S5C**). Interestingly, *ERF15* over-expression results in

delayed germination, similar to the phenotype we observed in *SUC2::CDF6* line (Lee et al., 2015). This suggests that vasculature-expressed *CDF6* may upregulate *ERF15* to delay germination and impact ABA responsiveness.

Next, we considered that a large number of DEGs could be generally cold responsive rather than differentially expressed as a result of the absence of CDF6. Thus, we compared the 524 DEGs (*SUC2::CDF6*) and 12 DEGs (*cdf6)* to our generally cold-responsive DEGs and identified 6 specific to *cdf6* and 440 DEGs specific to *SUC2::CDF6* with an additional six shared by both *SUC2::CDF6* and *cdf6* (**Figure 2.3D**). The twelve *cdf6* DEGs have various functions including defense (*AT3G59930, AT5G33355*), class 1 crossover (*HEI10*), methionine biosynthesis (*MRD1*), and metal transport (*IRT3*), in addition to a handful of genes with unknown function (Goto and Naito, 2002; Silverstein et al., 2005; Chelysheva et al., 2012; Lee et al., 2021); **Figure 2.3E**). To further assess the extent of misexpression due to the cold stress rather than the effect of *CDF6*, we also compared the 473, 344, and 1339 DEGs (**Figure 2.3A**) at 10ºC versus 22ºC in the WT, *cdf6*, and *SUC2::CDF6* genotypes, respectively. This analysis identified 61 DEGs unique to *cdf6* (**Supplementary Figure 2.S5D**), which are enriched for the following three biological GO terms: cellular response to heat, response to heat, and response to temperature stimulus (Supplementary Dataset 2.S3). While these DEGs are not generally cold-responsive in comparison to our WT dataset, they may be generally temperature responsive. The other *CDFs* are not differentially expressed in *cdf6* or *SUC2::CDF6* at either temperature, with the exception of *CDF3* downregulation in *SUC2::CDF6* at 10ºC (**Supplementary Figure 2.S5E)**. The data also confirms a significant depletion of *CDF6* mRNA in *cdf6* and elevation in *SUC2::CDF6*, both the response to cold and at ambient temperature, confirming this genotype elevates mRNA abundance in PCCs (**Figure 2.3E, Supplementary Figure 2.S5F and 2.S5G**). Future functional work with additional *cdf6* alleles and complementation lines is needed to define CDF6's regulatory mechanism.

In the 440 *SUC2::CDF6*-specific DEGs at 10ºC, we identified nine genes associated with flowering (*FT, FKF1, TEM2, GA3ox2, SOC1, PRR9, PRR5, FUL, BFT*), all of which are downregulated with the exception of *TEM2* (**Figure 2.3F**). This complements previous reports of reduced expression in *SUC2::CDF6* in the evening under ambient temperature conditions (Krahmer et al., 2019). Interestingly, a few clock or circadian-regulated genes, specifically *PRR9, PRR5,* and *FKF1*, are differentially expressed in *SUC2::CDF6* compared to WT at 10ºC indicating that there may be feedback regulation between *CDF6* and these components (**Figure 2.3F**). Many other flowering-related components identified are either TFs or involved in hormone signaling (Kinoshita and Richter, 2020; **Supplementary Dataset 2.S4**).

CDF6 alters the expression of key flowering genes during cold under long-day conditions

To better understand how *CDF6* regulates photoperiodic flowering during cold, we measured the abundance of the photoperiodic flowering component mRNAs, *FT*, *CO,* and *BFT*, via qRT-PCR over a 24 h period in continuous 10ºC or 22ºC long-day conditions. In WT and under constant cold, *FT* transcript accumulation is significantly increased from ZT20 to ZT36 and displays a shift in peak expression to ZT20 compared to its peak expression at ZT16 during ambient temperature (**Supplementary Figure 2.S6A**). In *SUC2::CDF6* lines, we observed significant reduction of *FT* transcript abundance relative to WT in the evening (ZT16, ZT20), at dawn (ZT24), and late afternoon (ZT32, ZT12, ZT36) (**Figure 2.4A**). At 10ºC, *FT* accumulation is reduced relative to WT throughout the 24 h period (**Figure 2.4B**). Interestingly, in *SUC2::CDF6*, *FT* repression was significantly enhanced during the cold at ZT16, further supporting a distinct role for CDF6 in regulating photoperiodic flowering during cold stress (**Figures 2.2A, 2.4C**).

Under ambient temperature, *CO* shows a shift in peak expression from ZT20 to ZT24 in *SUC2::CDF6* relative to WT and is significantly downregulated in *SUC2::CDF6* at ZT32 (**Figure 2.4D**). *CO* has peak expression at ZT20 under continuous cold, and is significantly repressed in the early morning (ZT20), at dawn (ZT24), and late evening (ZT12, ZT36) in *SUC2::CDF6* compared to WT (**Figure 2.4E**). In addition, *CO* is significantly upregulated in response to cold in WT at ZT24 so it is likely that *pSUC2*-driven expression of *CDF6* prevents *CO* accumulation during the cold at this time point (**Figure 2.4E, Supplementary Figure 2.S6B**). Although the repressive effect of *SUC2::CDF6* is not significantly different at 10ºC compared to 22ºC, our data together indicate that *CDF6* expression is required for appropriate accumulation of *CO* in WT at specific timepoints under both temperatures (**Figure 2.4F**). These findings are consistent with the transcriptome analysis, which shows that *FT* and *CO* are strongly downregulated during cold stress when *CDF6* is *pSUC2* regulated (**Figures 3D, 4**). *FT* and *CO* are not significantly different in *cdf6* compared to the WT at either temperature tested, likely due to redundancy by other *CDFs* in photoperiodic flowering regulation (**Supplementary Figure 2.S6C, 2.S6D**).

Besides *FT, CO* and the two clock components *PRR9* and *PRR5*, *BFT* is specifically localized to the PCC under control conditions (**Supplementary Figure 2.S7**). In addition, transcriptome analysis revealed *BFT* to be downregulated in *SUC2::CDF6* seedlings during cold stress (**Figure 2.3F**). Therefore, we also assessed *BFT* transcript accumulation during continuous cold under long-day conditions in *SUC2::CDF6*. At ambient temperature, *CDF6* downregulates *BFT* during the day (ZT12, ZT16, ZT28, and ZT36) (**Figure 2.4G)**. However during continuous cold, *BFT* transcript abundance is significantly reduced in *SUC2::CDF6* during both the day and night periods (ZT12 - ZT24 and ZT32) (**Figure 2.4F)**. Together, these data indicate that *CDF6* represses *BFT* independent of temperature, although *BFT* mRNA accumulation is significantly

increased in *SUC2::CDF6* in the early morning (ZT20 and ZT24) during continuous cold compared to ambient temperature (**Figure 2.4I)**.

Discussion

Previous work shows that the clock regulates a range of developmental programs including germination, flowering, and senescence to promote optimal survival and reproduction (Lu et al., 2012; Adams et al., 2018; Kim et al., 2018; Zha et al., 2019; Kyung et al., 2021). The clock is also known to regulate responses to abiotic stress. A classic example involves the clock gating of *CBF1-3* accumulation during cold stress, which promotes the activation of *COR* genes to confer increased cold tolerance (Fowler and Thomashow, 2002; Gilmour et al., 2004; Fowler et al., 2005). Additional evidence suggests that the expression of *CBFs* is regulated by PRR5, PRR7, and PRR9 (Nakamichi et al., 2009).

Here we investigate clock regulation of a poorly characterized member of the *CDF* family, *CDF6*, during ambient and cold temperatures. We find that CCA1 represses *CDF6* transcript accumulation during ambient temperature and gates the accumulation of *CDF6* during moderate cold stress (**Figure 2.5**). To better understand clock regulation of *CDF6*, we utilized the FIMO tool to scan the 500 bp upstream of each DOF-TF family member for motifs associated with clock TF binding (Grant et al., 2011). This analysis identified that proximal promoter regions of ~39% of the DOF-TFs contain a full or partial Evening Element or CCA1 Binding Site, whereas none of the DOF-TF promoters contain a primary G-box motif, the element which is associated with PRR binding (**Supplementary Dataset 2.S5**). This may explain the low number of DOF family members in PRR7 (~5%), PRR9 (~11%), and TOC1 (0) ChIP datasets compared to the CCA1 (~25%) and LHY (~14%) ChIP datasets, although it is important to note the CCA1 and LHY

datasets also identified many more target genes than the PRR datasets (Huang et al., 2012; Liu et al., 2013, 2016; Nagel et al., 2015; Kamioka et al., 2016; Adams et al., 2018); **Supplementary Dataset 2.S5**). We also considered that clock genes may not be the only upstream regulators of *CDF6*, therefore, we utilized the DAP-sequencing database to identify TFs that may bind to the *CDF6* promoter region (O'Malley et al., 2016). This revealed 142 unique gene IDs/TFs that bind *in vitro* along the *CDF6* promoter, gene body, or UTRs (**Supplementary Dataset 2.S6**). Of these, ~12% were DOF-TFs (including *CDF3, CDF4*, and *CDF5*) suggesting that these CDFs may regulate *CDF6* or other *DOF-TFs*. The finding that *CDF6* expression is significantly downregulated in *35S::CDF3* lines, further supports the conclusion that CDF3 modulates *CDF6* transcription (Corrales et al., 2017). Finally, ~20% of the TFs identified in the DAP-sequencing analysis were also significantly mis-regulated in our generally cold-responsive dataset (FDR < 0.05), implying that some of these TFs may also play a role in the cold regulation of CDF6 (**Supplementary Dataset 2.S6)**. Future work to investigate whether these TFs play a role in modulating the cold response of *CDF6* and other *DOFs* is needed.

The results of this study indicate that *CDF6* participates in seed germination regulation during ambient temperature and photoperiodic flowering during cold stress (**Figure 2.2**). Other DOF-TFs function in vascular system development and germination; however, with the exception of CDF4, the *CDFs* has not been previously associated with germination (Le Hir and Bellini, 2013). Our transcriptomic analysis revealed *IRT3*, *ERF15*, and 16 other DEGs may contribute to the observed germination phenotype (**Figure 2.5, Supplementary Figure 2.S4**). Of note, our dataset was generated under a later developmental stage than when germination was phenotypically assessed, thus future work at the same developmental stage could elucidate any additional DEGs that may contribute to the delayed germination in *SUC2::CDF6*.

Our study supports a greater role for *CDF6* function under cold versus ambient temperature to broadly control plant growth and development. We identified a number of flowering associated genes that have altered expression in vasculature-expressed *CDF6* during cold stress, four (*FT*, *BFT*, *PRR9*, *PRR5*) of which are also expressed in the PCC **(Figure 2.3, Supplementary Figure 2.S7A-D)** (Mustroph et al., 2009). Of note, single cell sequencing data in Arabidopsis shows that *CDF6* may also be expressed in phloem parenchyma, xylem and even mesophyll cells (Mustroph et al., 2009; Kim et al., 2021). We also identified a number of GO terms associated with responses to stress, development, and metabolism (**Figure 2.3**). Whereas the response to stress and development GO terms corroborate our earlier findings for *CDF6* during cold stress and in relation to both the observed germination and flowering phenotypes, the metabolism GO terms are interesting as other CDFs have been previously implicated in metabolic regulation. A transcriptomic analysis of *35S::CDF3* indicates enrichment of metabolism GO terms, and more specifically, a metabolic analysis indicates that *CDF3* over-expression impacts metabolism of sugars and amino acids (Corrales et al., 2017). While the results of the GO analysis for both *SUC2::CDF6* and *35S::CDF3* yielded similar enriched terms, only a single gene is shared between the DEGs in the *CDF3* (531) and *CDF6* (18) datasets. The shared gene, *AT4G01390*, is significantly downregulated in *SUC2::CDF6* and upregulated in *35S::CDF3*. Based on public annotation, *AT4G01390* contains a MATH [meprin and TRAF (tumour necrosis factor receptor-associated factor) homology] domain which are found in proteins involved in several of the GO terms shared between the two datasets such as stress responses, plant development, signaling, and metabolism (Oelmuller et al., 2005; Inzé et al., 2012; Qi et al., 2021). Of note, the lack of significant overlap between the two datasets is not surprising given the differences in tissue-specific expression (*35S::CDF3* vs *SUC2::CDF6*), experimental design, and analysis. Together, this indicates that

CDF3 and *CDF6* may work similarly but independently to integrate temperature signals to alter metabolism and development.

Next, we observed that vasculature-expressed *CDF6* results in altered *FT, CO,* and *BFT* accumulation across the 24 h period at both 22°C and 10°C, but notably during continuous cold in long-days (**Figure 2.4**). Specifically, we found that *FT* has higher accumulation during the cold from ZT20 – ZT36, while *CO* is higher at ZT24 and lower at ZT32 (**Supplementary Figure 2.S6)**. At first glance, this seems contrary to previous work, which indicates that lower temperatures lower *FT* accumulation and delayed flowering (Song et al., 2013). However, the impact of temperature on *FT* accumulation is highly dependent on the timing of the temperature stress and the photoperiod length (Kinmonth-Schultz et al., 2016; Krahmer et al., 2019). For example, when plants are grown in ambient long-days with cool nights, *CO* has higher transcript accumulation at dawn (ZT0) and no change at midday (ZT8) or dusk (ZT16), while *FT* is higher at dawn with a decrease or no change at midday and dusk as compared to the constant ambient temperature control condition (Kinmonth-Schultz et al., 2016). In constant light, *FT* exhibits increased transcript abundance during cold treatment (Schwartz et al., 2009). *FT* is dynamically controlled during cold temperature exposure by multiple regulators including *FLC, SHORT VEGETATIVE PHASE (SVP), HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES (HOS1), miR156, miR172*, and sequestration of FT through its interaction with phospholipid phosphatidylglycerol (PG) at cellular membranes (Song et al., 2013; Susila et al., 2021). Our study shows for the first time that a *CDF* family member contributes to the differential transcript accumulation of key photoperiod regulators, *FT, CO,* and *BFT* during cold stress. Additionally, ectopic expression of *CDF6* with *pSUC2* results in differential expression of 34 and 530 downstream genes under ambient and cold temperatures, respectively. (**Figure 2.5**). We conclude that CDF6 directly or indirectly regulates transcription of numerous genes, particularly at low temperatures.

Taken together, our data suggest that vasculature-expressed *CDF6* play a role in regulating photoperiodic flowering during cold stress, and some of this regulation involves functional redundancy with other *CDFs*. We provide new insights on the regulatory relationship between *CDF6* and the clock, cold stress, and plant development. As climate change continues to cause erratic weather events, the precise regulation of photoperiod flowering components in specific cell-types during cold temperature should be further explored as a tool to combat potential crop losses.

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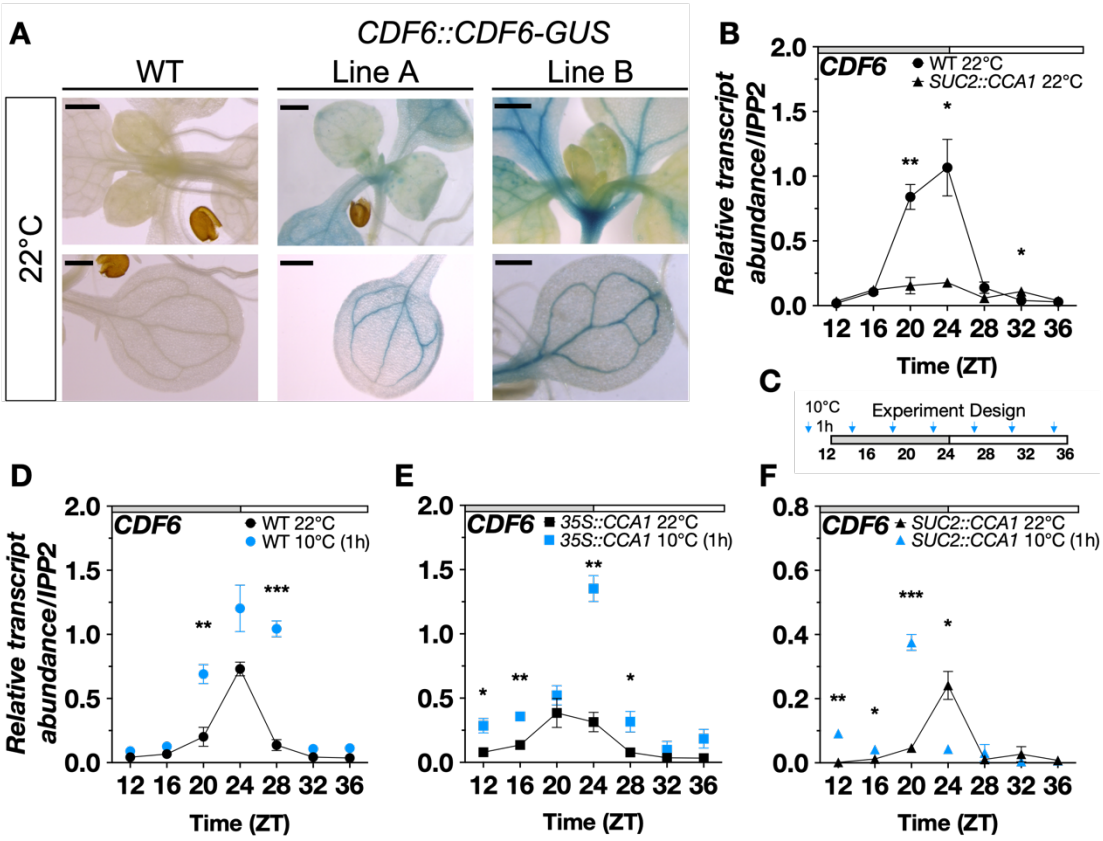
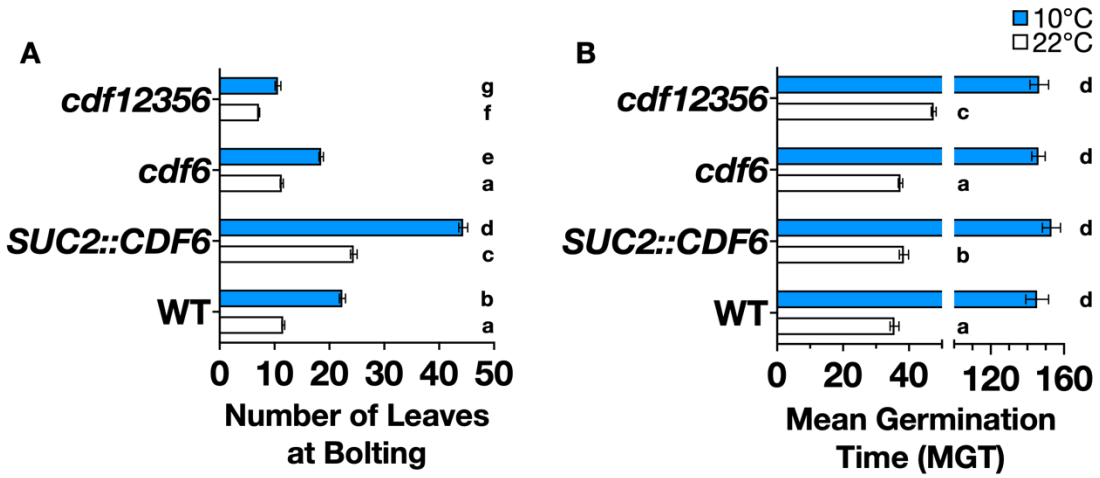
Figures

Figure 2.1: Vasculature-specific CDF6 expression is gated by the clock in response to cold stress.



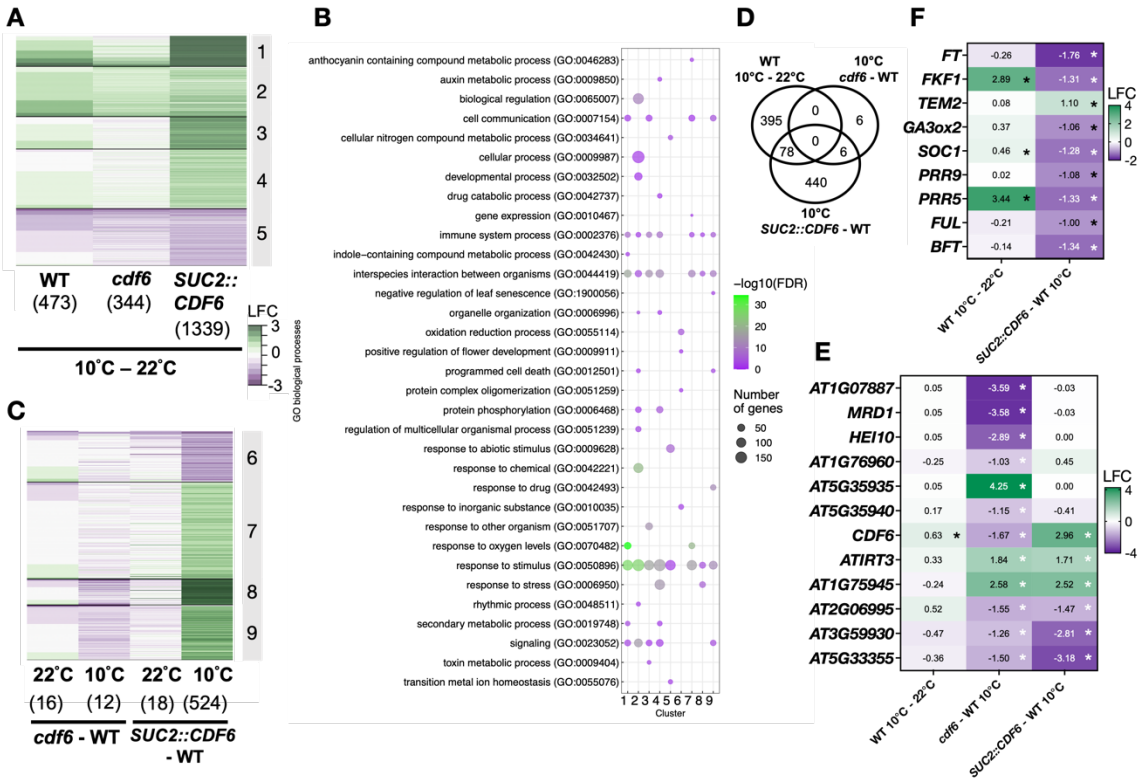
(A) GUS activity in WT and two independent T3 *CDF6::CDF6-GUS* plants (Line A and B) indicate the expression pattern of *CDF6* at ZT0 in 11 day old seedlings grown in long-day (16 h light: 8 h dark) conditions. Bars correspond to 500 µm. **(B)** qRT-PCR of *CDF6* transcript abundance in WT (Col-0) and *SUC2::CCA1* in ambient temperature. qRT-PCR of *CDF6* transcript abundance in **(C)** continuous 22°C or 1 h of 10°C exposure in **(D)** WT, **(E)** *35S::CCA1*, and **(F)** *SUC2::CCA1*. Seedlings were grown in continuous light (LL) for 2 days after 8 days of entrainment in diurnal (12 h light:12 h dark;LD) cycles. Gray and white bars indicate the subjective night and day periods, respectively. Time (ZT) represents hours. mRNA levels are normalized to *IPP2* (mean values ± Standard Error (SE), n=3; ***P ≤ 0.001, **P ≤ 0.05, *P ≤ 0.01; unpaired student t-test).

Figure 2.2: *CDF6* mis-expression alters flowering and seed germination.



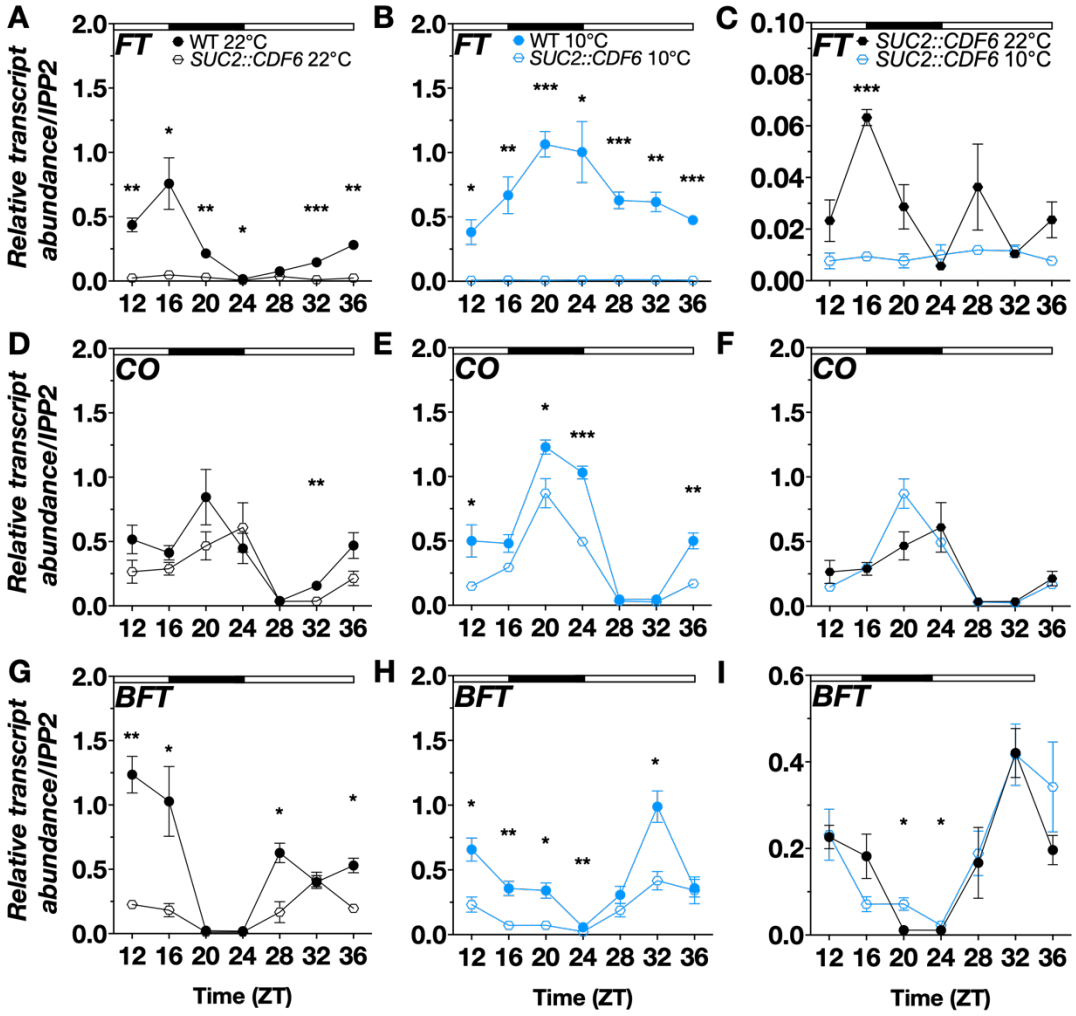
(A) Number of leaves at bolting for WT, *SUC2::CDF6, cdf6*, and *cdf12356* plants grown in constant 22°C (white bars) or 10°C (blue bars) in long-day conditions (mean values ± SE, n=3+, 2-way ANOVA with Tukey's multiple comparison test). (B) Mean germination time (MGT) for WT, *SUC2::CDF6, cdf6,* and *cdf12356* plants grown in constant light (LL) at 10°C or 22°C with a-c indicating statistical significance at 22°C and d at 10°C (mean values ± SE, n=4, 1-way ANOVA with Dunnett's multiple comparison test).

Figure 2.3: Vasculature-expressed *CDF6* regulates cold-responsive and flowering-associated genes in the morning.



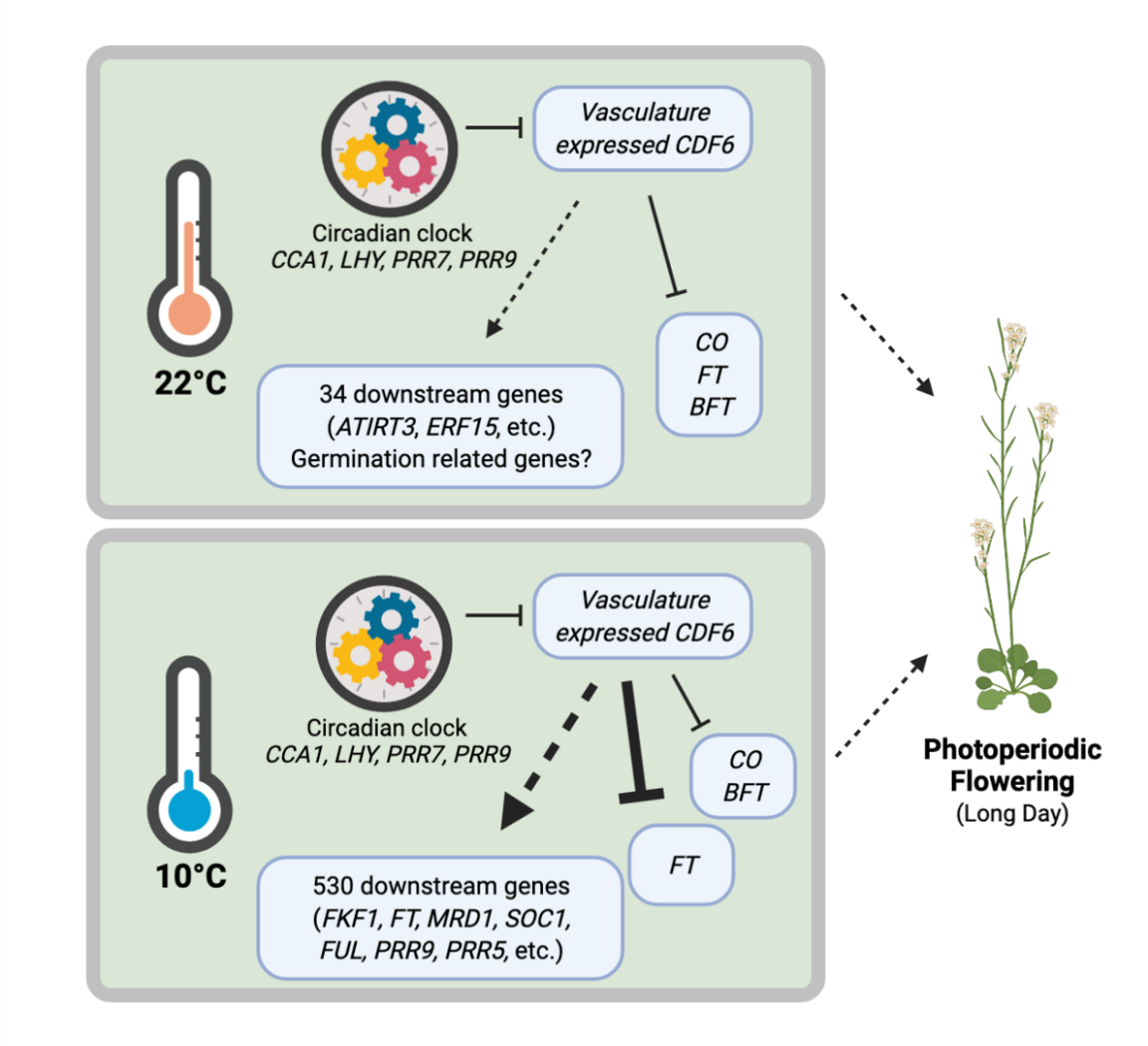
(A) Temperature and **(C)** genotype-specific DEGs [defined as -1 > Log_2 Fold Change (LFC) > 1 and False Discovery Rate (FDR) < 0.05] identified after RNA-sequencing of three replicates of 11 day old WT, *SUC2::CDF6,* and *cdf6* seedlings. Seedlings were grown in constant light for 2 days at 22˚C after entrainment for 8 days in diurnal (LD) conditions before sampling at ZT0 after 1 h at 10˚C or continuing at 22˚C. **(B)** Summary of enriched GO biological process terms with indicated False Discovery Rate (FDR corrected p-value < 0.05) GO terms selected based on a previously published protocol using a FDR cut-off < 0.05 (Bonnot et al., 2019). **(D)** DEGs specific to the cold response versus *CDF6* mis-regulation during cold stress. Expression profile of **(E)** 12 DEGs mis-expressed in *cdf6* and **(F)** flowering-associated genes identified in *SUC2::CDF6* during cold stress; *FDR < 0.05.

Figure 2.4: CDF6 alters *FT*, *CO,* and *BFT* expression during cold stress.



qRT-PCR of **(A-C)** *FT*, **(D-F)** *CO,* and **(G-I)** *BFT* relative transcript abundance in WT and *SUC2::CDF6* seedlings. Plants were grown in long-day conditions at 22˚C for 8 days followed by 2 days at continuous 22˚C or 10˚C. Seedlings were sampled every 4 h starting at 12 h after dawn. **(C)** *FT* **(F)** *CO,* and **(I)** *BFT* transcript abundance in *SUC2::CDF6* at 10˚C and 22˚C from the data shown in A and B, D and E, and G and H, respectively. White and black bars indicate the day and night periods, respectively. mRNA levels are normalized to *IPP2* (mean values ± SE, n=3; ***P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05; unpaired student t-test).

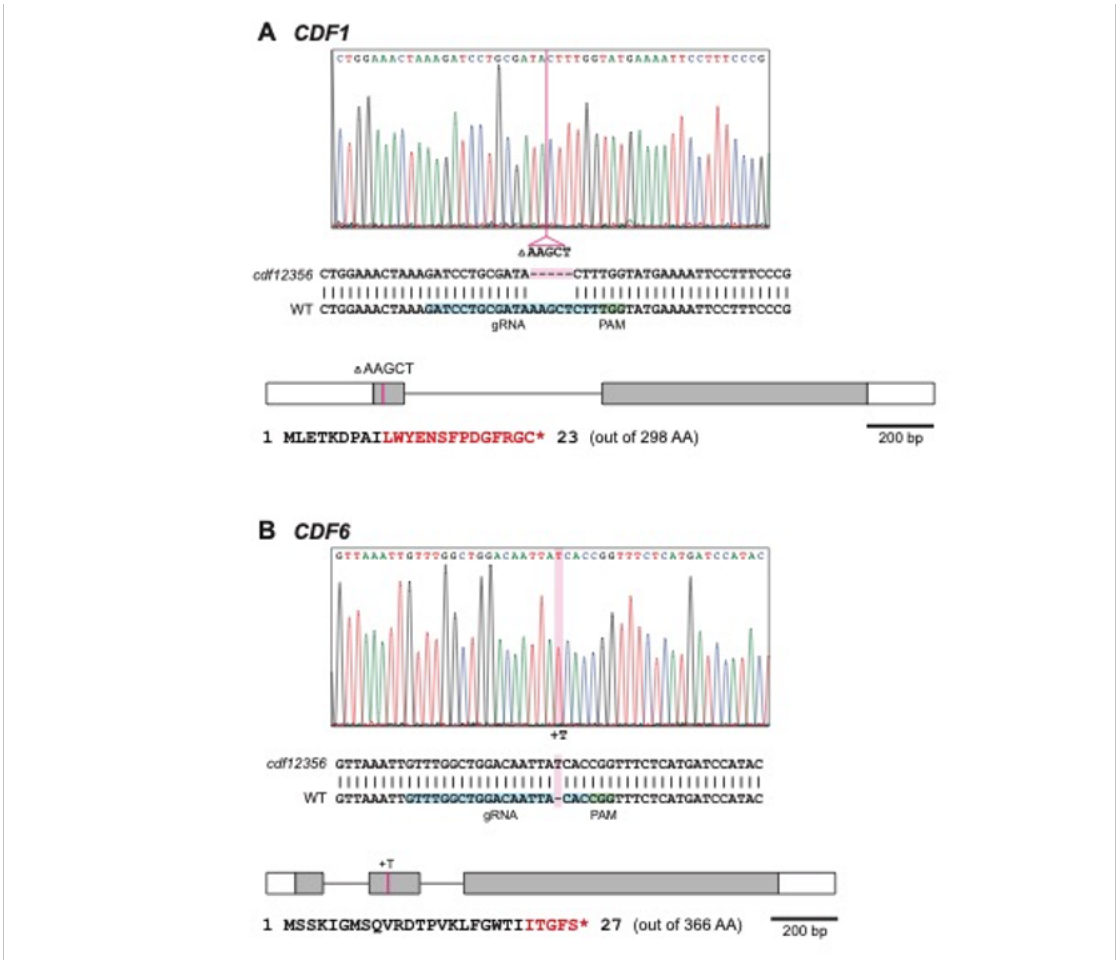
Figure 2.5: Clock regulated, cold induced, and vasculature expressed *CDF6* fine-tunes plant growth and development under different temperatures.



The circadian clock regulates *CDF6* transcript accumulation in the vasculature at specific times of day. During ambient temperature, *CDF6* regulates *FT, CO, BFT* and at least 34 other downstream genes (16 in *cdf6*-WT and 18 in *SUC2::CDF6*-WT) including *ATIRT3* and *ERF15,* which contribute to germination and flowering phenotypes. During cold temperatures, *CDF6* regulates 530 downstream genes and represses *FT, CO,* and *BFT* in the vasculature/phloem companion cells to control photoperiodic flowering. Model created with BioRender.com.

Supplementary Figures and Tables

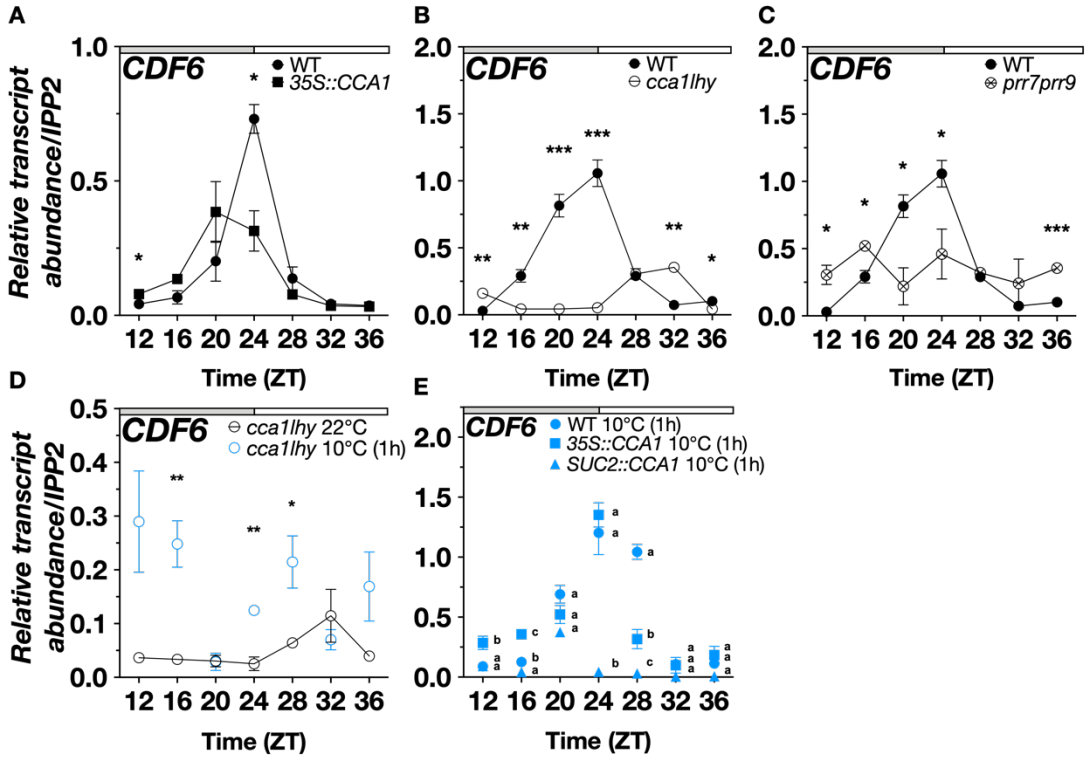
Supplementary Figure 2.S1: CRISPR/Cas9-induced mutations in *CDF1* and *CDF6* in the *cdf12356* mutant.



A) A chromatogram of the region where mutations exist in the *CDF1* gene in the *cdf12356* mutant is presented. The nucleotide alignment of the same regions between the *cdf12356* and WT is shown under the chromatogram. The position of 5-base-pair deletion in *CDF1* in the *cdf12356* is highlighted in pink in the alignment. The positions of the gRNA and the PAM sequences are also highlighted. The position of the mutation is indicated in the diagram of the *CDF1* gene structure. The white and gray boxes represent UTRs and exons, respectively, and the bars connecting boxes are introns. The deduced amino acid sequences in the *cdf12356* are shown. The amino acid sequences that differed from the WT CDF1 sequences are highlighted in red. **(B)** The information regarding the mutation in *CDF6* in the *cdf12356* mutant. The chromatogram of the mutation in CDF6 in the *cdf12356* mutant, the nucleotide sequence alignment, the schematic diagram of the

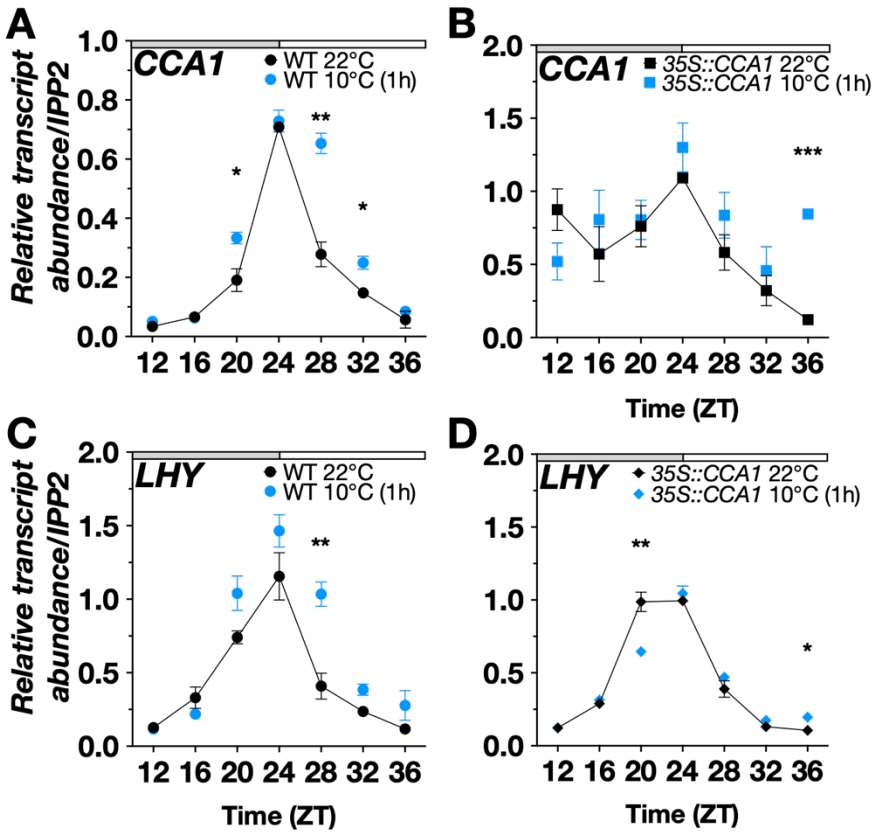
CDF6 gene, and the deduced amino acid of resulting CDF6 protein are shown. The detailed information of the figures is the same as that of **(A)**.

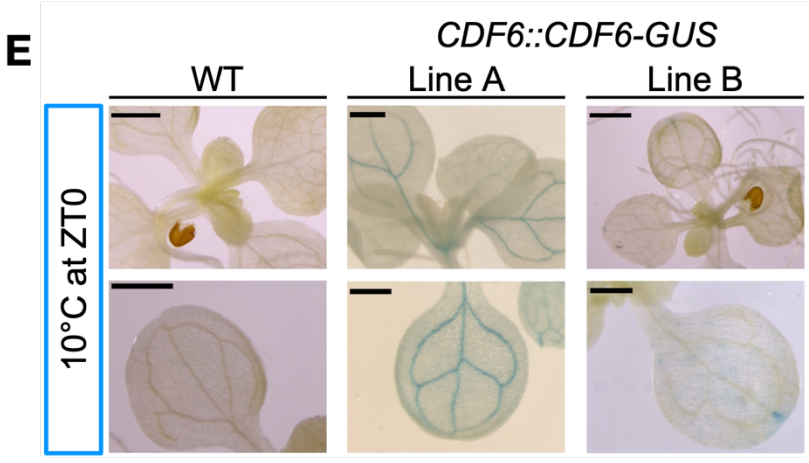
Supplementary Figure 2.S2: *CDF6* is regulated by morning and day-phased clock components.



qRT-PCR of *CDF6* in WT, **(A)** *35S::CCA1*, **(B)** *cca1lhy*, and **(C)** *prr7prr9* seedlings grown in constant light for 2 days after 8 days entrainment in 12 h light:12 h dark (LD) cycles. Plants were sampled every 4 h for 24 h. qRT-PCR of CDF6 in **(D)** *cca1lhy* in continuous 22˚C after 1 h pulse of 10˚C grown in constant light for 2 days after 8 days entrainment in LD. **(E)** qRT-PCR of *CDF6* pulse of 10˚C in WT, *35S::CCA1*, and *SUC2::CCA1* from the gating experiment shown in Figure 2.1D-F. mRNA levels are normalized to *IPP2* (mean values ± SE, n=3, ***P ≤ 0.001; **P ≤ 0.01; *P ≤ 0.05, unpaired student t-test).

Supplementary Figure 2.S3: *CCA1* and *LHY* have time of day specific response to cold stress and CDF6 protein localizes to vasculature during cold stress.

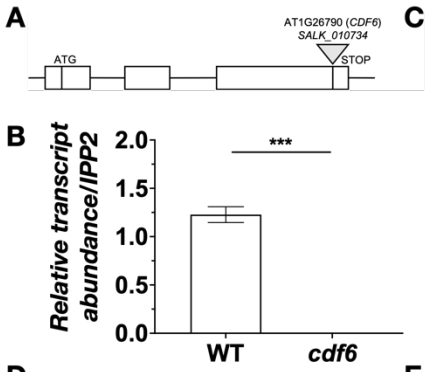




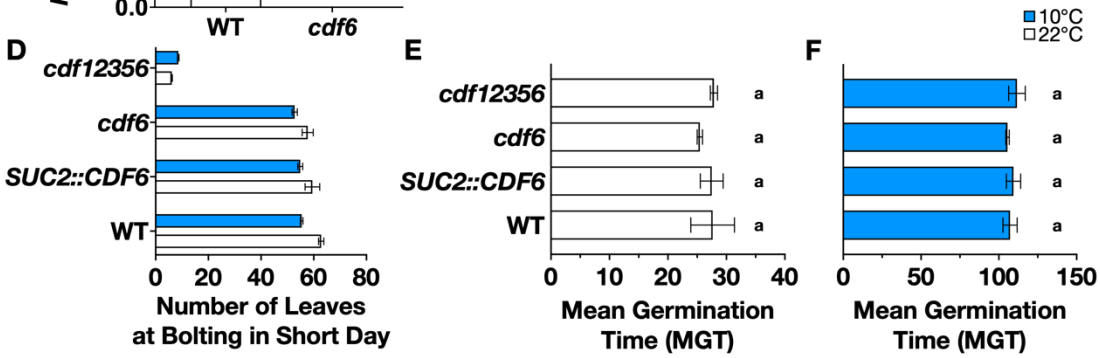
qRT-PCR of **(A and B)** *CCA1* and **(C and D)** *LHY* transcript levels in WT (Col-0) and *35S::CCA1* seedlings grown in constant light for 2 days after 8 days entrainment in 12 h light:12 h dark (LD) cycles. Plants were sampled every 4 h for 24 h at constant 22°C or after a 1 h 10°C exposure.

mRNA levels are normalized to *IPP2* (mean values ± SE, n=3, ***P ≤ 0.001; **P ≤ 0.01; *P ≤ 0.05, unpaired student t-test). **(E)** GUS activity indicates expression pattern of CDF6 at ZT0 in 11 day old seedlings grown in long-days and then exposed to 1 h 10°C treatment. Bars correspond to 500 μm.

Supplementary Figure 2.S4: *CDF6* transcript abundance is downregulated in *cdf6* and dormancy does not change in *CDF6* mis-expression lines.

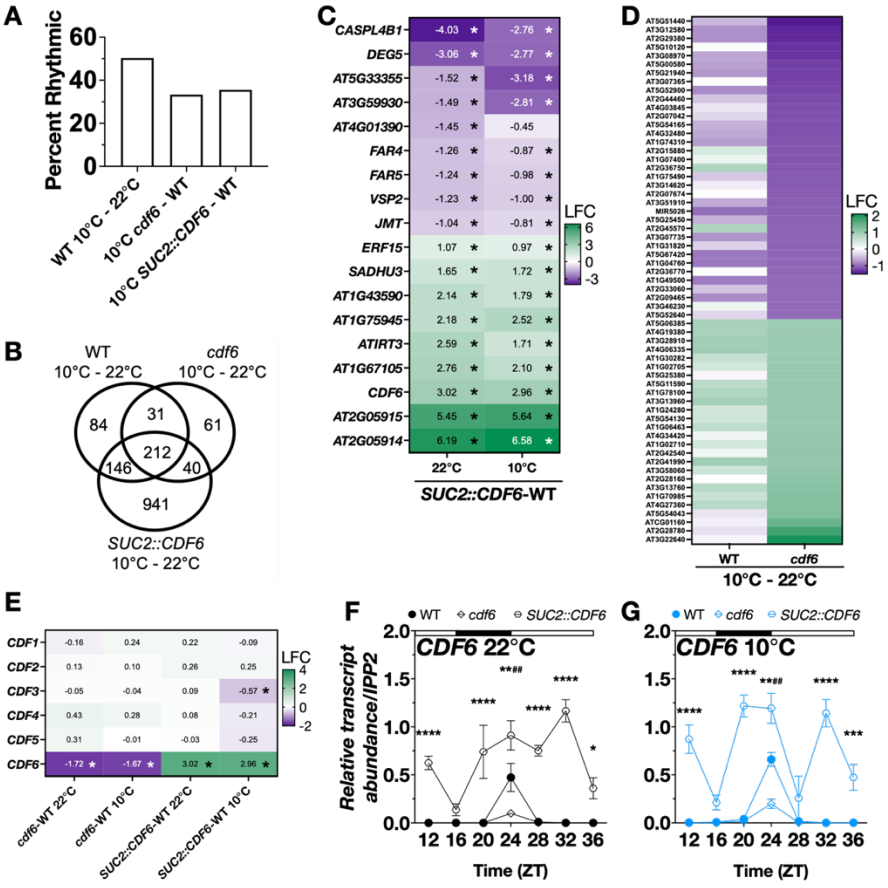


ANOVA of Interaction of Genotype and Temperature on Flowering			
Source of Variation	**% of total variation**	**P value**	**P value summary**
Interaction	6.185	<0.0001	****
Genotype	65.91	<0.0001	****
Temperature	21.46	<0.0001	****



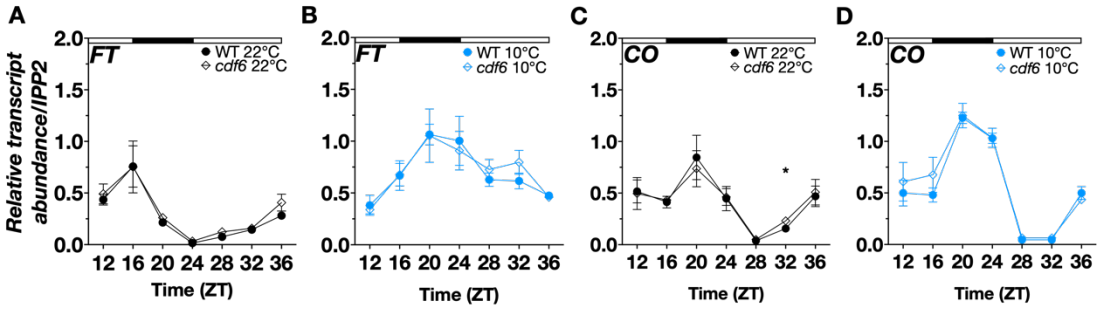
(A) Gene model with SALK T-DNA insertion mutant line (SALK_010734) for *CDF6* based on Araport11. **(B)** qRT-PCR of *CDF6* transcript accumulation in 10 day old seedlings grown in 12 h light:12 h dark (LD) cycles and sampled at ZT0. mRNA levels are normalized to *IPP2* (mean values ± SE, n=3, ***P ≤ 0.001; **P ≤ 0.01; *P ≤ 0.05, unpaired student t-test). **(C)** Results of the ordinary 2-way ANOVA performed on Figure 2.2A data. (**D**) Number of leaves at bolting for WT, *SUC2::CDF6*, *cdf6* and *cdf12356* plants grown in constant 22°C or 10°C in short day conditions (mean values ± SE, n=1). Mean germination time for WT, *SUC2::CDF6, cdf6* and *cdf12356* plants grown in constant light after 2-3 nights of stratification **(E)** 22°C and **(F)** 10°C (mean values ± SE, 1-way ordinary ANOVA with Dunnett's multiple comparison test).

Supplementary Figure 2.S5: Genome-wide expression analysis reveals both cold-dependent and *CDF6*-dependent DEGs.



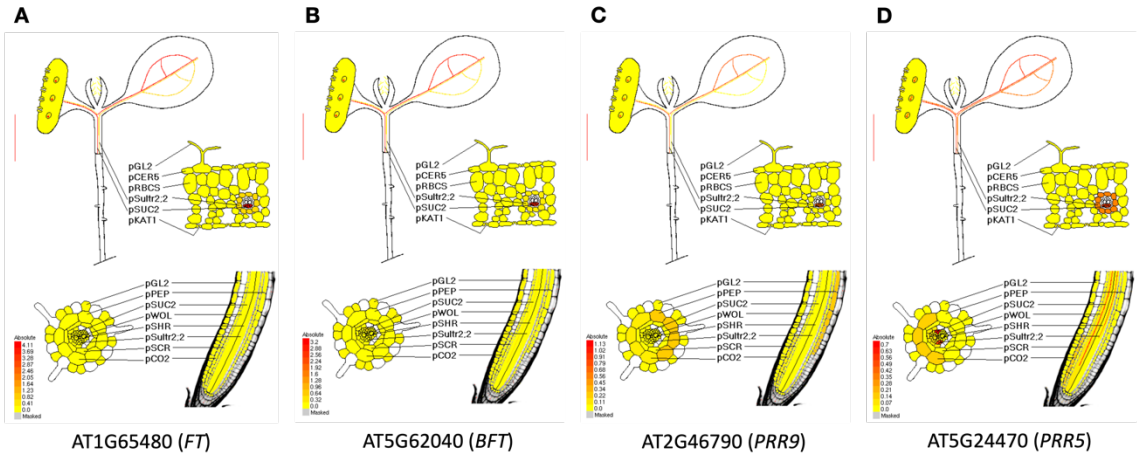
(A) Percentage of DEGs (defined as -1 > Log_2 Fold Change (LFC) > 1 and False Discovery Rate (FDR) < 0.05) that exhibit rhythmic pattern of expression determined using the Phaser webtool (Mockler et al., 2007). (**B**) Number of shared and unique DEGs at 10°C versus 22°C in WT, *SUC2::CDF6,* and *cdf6*. LFC for **(C)** 18 genes that are differentially expressed in *SUC2::CDF6* compared to the WT at 22°C, for **(D)** 61 genes differentially expressed in *cdf6* at 10°C compared to 22°C, and for **(E)** *CDF1, 2, 3, 4, 5,* and *6* in *cdf6* or *SUC2::CDF6* compared to WT at both 10°C and 22°C (*FDR < 0.05). qRT-PCR of *CDF6* transcript accumulation in 11 day old seedlings grown in long-day (16 h light: 8 h dark) at 22°C for 8 days followed by 2 days maintained at continuous **(F)** 22°C or transferred to continuous **(G)** 10°C. Plants were sampled every 4 h for 24 h. mRNA levels are normalized to *IPP2* (mean values ± SE, n=3, ****P ≤ 0.0001; ***P ≤ 0.001; **/##P ≤ 0.01; *P ≤ 0.05; where * indicates a significant difference between WT and *SUC2::CDF6* while # indicates a significant difference between WT and *cdf6*

Supplementary Figure 2.S6: FT and CO response to cold in WT and *cdf6*.



qRT-PCR of **(A, C)** *FT* and **(B, D)** *CO* relative transcript abundance in **(A-B)** WT and **(C-D)** *cdf6* seedlings grown in long-day (16 h light: 8 h dark) conditions at 22˚C for 8 days followed by 2 days at continuous 22˚C or 10˚C. Seedlings were sampled every 4 h starting at 12 h after dawn. mRNA levels are normalized to *IPP2* (mean values ± SE, n=3; ***P ≤ 0.005, **P ≤ 0.01, *P ≤ 0.05; unpaired student t-test).

Supplementary Figure 2.S7: Flowering and clock transcripts primarily localize to the vasculature/phloem companion cells (pSUC2) in control conditions.



(A) *FT*, (B) *BFT*, (C) *PRR9*, and (D) *PRR5* transcripts primarily localize to the vasculature/phloem companion cells (pSUC2) in control conditions. Pictograms were taken from http://efp.ucr.edu/ (Mustroph et al., 2009). Data represent signal values from microarray; scale bars are different between panels.

Supplementary Table 2.S2: PCR Conditions and Primer Sequences used in RT-qPCR

RT-qPCR conditions were 95°C for 3 min, 40 cycles at 95°C for 15 sec and 60 °C for 1 min, 95°C for 10 sec, and melt curve from 65°C to 95°C with 0.5°C increments. *Isopentenyl-diphosphate delta-isomerase II (IPP2)* was used as the normalization control.

LHY-F: 5'-CAACAGCAACAACAATGCAACTAC-3'

LHY-R: 5'-AGAGAGCCTGAAACGCTATACG-3'

CCA1-F: 5'-CAGCTCCAATATAACCGATCCAT-3'

CCA1-R: 5'-CAATTCGACCCTCGTCAGACA-3'

CDF6qPCR-F: 5'-GACTTGTATTGTCAGTAACAGATTGG-3'

CDF6qPCR-R: 5'-TGGCTGGACAATTACACCG-3'

CDF6qPCR-F:* 5' -GACATTACACTTCAGCATTTCCA -3'

CDF6qPCR-R:* 5' -ATCTATCTTATTTATATACCACAATCCC -3'

IPP2-F: 5'-GTATGAGTTGCTTCTGGAGCAAAG-3'

IPP2-R: 5'-GAGGATGGCTGCAACAAGTGT-3'

CO-F: 5'-CTACAACGACAATGGTTCCATTAAC -3'

CO-R: 5'-CAGGGTCAGGTTGTTGC -3'

FT-F: 5'-CTGGAACAACCTTTGGCAAT -3'

FT-R: 5'-TACACTGTTTGCCTGCCAAG -3'

CDF6pro-F: 5'-CACCGTTCTGTTTCAGAAGCAAGAATTT -3'

CDF6cds-R: 5'-GGCAAGATCTATGAACTTCAGAGA -3'

SALK_010734-F: 5' -TCGGATTTTGAAAGGTTGTTG - 3'

SALK_010734-R: 5' -GTTACTTCCTCCCCAAGCATC - 3'

SALK_010734qPCR-F: 5'- GACATTACACTTCAGCATTTCCA -3'

SALK_010734qPCR-R: 5'- ATCTATCTTATTTATATACCACAATCCC -3'

List of Supplementary Files/Datasets

Supplementary Dataset 2.S1: List of All DEGs

Supplementary Dataset 2.S2: *COR* Genes

Supplementary Dataset 2.S3: Phaser Results

Supplementary Dataset 2.S4: Flowering Genes

Supplementary Dataset 2.S5: DOF family members meta-data analysis

Supplementary Dataset 2.S6: DAP-sequencing Results

Chapter 3

PLATZ transcription factors are regulated by the circadian clock during abiotic stress

Abstract

In *Arabidopsis,* the circadian clock regulates a large proportion of heat-responsive genes, which allows plants to better withstand extreme heat stress. Here, we examine the *PLATZ* family of transcription factors, which has been largely unexplored in relation to both the clock and heat stress. We found evidence for clock regulation of about half the *PLATZ* family members, while more than half of the *PLATZ* family members respond to temperature stress. We found that *AT1G32700* and *AT5G46710* are constitutively induced in response to heat stress, while *PLATZ2* responds to heat at specific times of day. To better understand the broad function of *PLATZ* genes during heat stress, we performed a heat shock assay and transcriptome analysis. Lastly, after identifying *PLATZ* orthologs in sorghum and rice, we discovered that one *SbPLATZ* gene responds to heat stress at multiple times of day and obtained evidence that multiple *OsPLATZ* genes may be clock-regulated.

Introduction

The circadian clock is a regulatory network with a period of ~24 h that allows plants and other organisms to synchronize daily metabolism, physiology, growth, and development with environmental changes (McClung, 2019). Environmental stimuli, such as light and temperature, serve as inputs that can entrain the circadian clock (Nohales and Kay, 2016; Sanchez and Kay, 2016). The clock perceives inputs and then outputs them as rhythms which can be observed in physiology, photosynthesis, and response to abiotic stress, among many others (Nohales and Kay, 2016; Sanchez and Kay, 2016). The core clock integrates environmental signals through a series of negative transcriptional-translational feedback regulation loops (Nagel and Kay, 2012; Nohales

and Kay, 2016; Sanchez and Kay, 2016). The classical feedback loop involves the partially redundant, morning-expressed MYB transcription factors, *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY),* repressing the transcription of evening-expressed *TIMING OF CAB 1* (*TOC1*). (Alabadí et al., 2001). *PSEUDO RESPONSE REGULATOR9* (*PRR9*) and *PRR7* form another arm of the morning loop in which they repress and are repressed by *CCA1* and *LHY* (Nakamichi et al., 2007). The *PRRs* (*PRR9, PRR7, PRR5, PRR3, PRR1/TOC1*) are expressed sequentially throughout the day from early afternoon to evening (Nakamichi et al., 2007). *CCA1* and *LHY* also form a negative feedback loop with the Evening Complex (EC), which is comprised of a MYB-like transcription factor, *LUX ARRYTHMO* (*LUX*), *EARLY FLOWERING 3* (*ELF3*), and *ELF4* (Nagel and Kay, 2012; Sanchez and Kay, 2016). *TOC1*, *CCA1*, and *LHY* repress *GIGANTEA (GI)*, which is an important clock component that regulates numerous outputs including light signaling and flowering among others (Sawa et al., 2007; Nohales et al., 2019).

The circadian clock allows plants to anticipate and respond to cyclical environmental stimuli through various output mechanisms such as the response to stress (Grundy et al., 2015). For example, heat stress is more likely to occur in the afternoon, thus the clock can leverage this environmental pattern to synchronize an effective heat stress response evidenced by the large proportion of heat-responsive genes (~50%) that are also clock regulated (Covington et al., 2008; Grundy et al., 2015). As many core clock components are transcription factors, an important way for the clock to control this large proportion of heat-responsive genes is through transcriptional activation or repression of downstream genes. Morning expressed *CCA1* regulates about 2000 genes based on published ChIP-sequencing (ChIP-seq) data, which further demonstrates the opportunity for a clock TF to cause a transcriptional cascade to regulate the expression of a large subset of genes (Nagel et al., 2015).

My previous work (Chapter 1) investigated how the clock through *CCA1, LHY, PRR7,* and *PRR9* regulate the transcriptome during temperature stress (Blair et al., 2019). Through that analysis, we identified a subset of candidate genes, including *PLATZ2*, that are clock-regulated and heat-responsive. *PLATZ2* belongs to the plant-specific *PLATZ* (plant AT-rich sequence-and zinc-binding protein) family of transcription factors (Blair et al., 2019). The *PLATZ* family was first identified in peas, and the *PLATZ* domain is characterized by two zinc binding motifs (Nagano et al., 2001). Electrophoretic mobility shift assay indicates that pea PLATZ1 binds to A/T rich region sequences, and a luciferase (LUC) reporter assay using the *35S* promoter to drive expression of LUC fused to a tandem repeat of the A/T rich sequence resulted in decreased LUC activity suggesting that PLATZ transcription factors act as general transcriptional repressors (Nagano et al., 2001). *PLATZ2* and its closely related ortholog, *PLATZ1*, have been previously shown to respond to multiple abiotic stresses (González-Morales et al., 2016; Liu et al., 2020). For example, *PLATZ1* and *PLATZ2* were identified as nodes in a desiccation tolerance network (González-Morales et al., 2016). More specifically, transgenic *PLATZ1* overexpression lines exhibit tolerance to low water stress (González-Morales et al., 2016). *PLATZ2* is induced by salt stress, and ChIP quantitative real-time PCR (qRT-PCR) demonstrates that PLATZ2 associates with *SOS3* and *SCaBP8* promoter regions to inhibit their transcription and suppress plant salt tolerance (Liu et al., 2020). *PLATZ* family members have also been linked to early plant development. For example, *platz2* displays delayed germination (González-Morales et al., 2016). Another *PLATZ* family member, *ORESARA15* (*ORE15*; *AT1G31040*) suppresses leaf senescence by modulating the *GROWTH REGULATING FACTOR* (*GRF*)/*GRF*-interacting factor regulatory pathway (Kim et al., 2018). Transgenic plants carrying an *ore15* dominant mutation show delayed senescence and increased plant height, root length, and seed weight (Kim et al., 2018). Additionally, RNA-sequencing (RNA-seq) analysis indicates that *ORE15* increases the rate and

duration of cell proliferation to enhance leaf growth (Kim et al., 2018). *ORE15* is also expressed in the root apical meristem (RAM), where it mediates the interaction between auxin and cytokinin to control RAM size (Timilsina et al., 2022).

Phylogenetic analyses of the *PLATZ* transcription factors have been done in many other species including rice, maize, soybean, wheat, buckwheat, and *Brassica rapa* (Holmes, 2017; Wang et al., 2018; Azim et al., 2020; Fu et al., 2020; He et al., 2021; Li et al., 2022; Zhao et al., 2022). Many non-*Arabidopsis* family members are also involved in abiotic stress responses and are specifically induced during drought, salt, ABA, or GA treatment (So et al., 2015; Zhang et al., 2018; He et al., 2021). In soybean, GmPLATZ17 interacts with GmDREB5 resulting in reduced DRE binding and subsequent repression of downstream genes to inhibit the drought tolerance response (Zhao et al., 2022). *GmPLATZ1* overexpression lines are susceptible to ABA and show delayed germination during osmotic stress (So et al., 2015).

Rice and maize *PLATZ* family members have also been implicated in regulating development and other fundamental molecular processes. In maize, FLOURY3 (FL3; ZmPLATZ12) interacts with RPC53 (RNA polymerase III subunit C53) and TFC1 (transcription factor class C1), two components of the RNA polymerase III (RNAPIII) transcription complex (Li et al., 2017). Fourteen other maize PLATZ family members associate with either RPC53 or TFC1 indicating that this family plays a general role in small non-coding RNA transcription through interaction with RNAPIII components (Wang et al., 2018). *ZmPLATZ6*, the most closely related maize ortholog to *AtPLATZ2*, interacts with both RPC53 and TFC1 (Wang et al., 2018). In rice, *osgl6*, which is most closely related to *ORE15* in *Arabidopsis*, shows decreased grain size, increased panicle length, and is expressed highly in young panicles (Wang et al., 2019). Similarly, *short grain 6 (ossg6)* mutants show reduced grain weight and length and are also expressed in early developing panicles (Zhou and Xue, 2020). OsGL6 may participate in

ribosome biogenesis through its association with the RNAPIII transcription machinery evidenced by the interaction of OsGL6 with OsRPC53 and OsTFC1, while OsSG6 may control grain size by impacting spikelet hull cell division through interaction with cell cycle and cell division regulators (Wang et al., 2019; Zhou and Xue, 2020). There is also evidence that *PLATZ* family members in wheat, buckwheat, and *Brassica rapa* are also involved in regulating development (Azim et al., 2020; Fu et al., 2020; Li et al., 2022). Considering the broad functions of *PLATZ* family members in regulating responses to stress in addition to plant growth and development across many species, it will be interesting to further characterize *PLATZ2* and its family members.

Here we investigate how *Arabidopsis PLATZ2* and other *PLATZ* family members may be heat-responsive or clock controlled. To better understand clock regulation of *PLATZ* family members, we performed a meta-data analysis and identified five cycling *PLATZ* family members. We also found that *PLATZ* family members respond to abiotic stresses including heat stress, cold stress, drought, and hypoxia. Some *PLATZ* family members are constitutively induced during heat stress, while *PLATZ2's* heat induction is dependent on time of day. Next, we examined *PLATZ2* more closely to determine its impact on the heat stress response through heat shock assays and transcriptomic analysis. We also investigated orthologs of *Arabidopsis PLATZ* genes in rice (*Oryza sativa*) and sorghum (*Sorghum bicolor*), and we identified 11 cycling rice *PLATZ* orthologs and one heat-responsive sorghum *PLATZ* ortholog. To our knowledge, this study is one of the first to indicate that rice *PLATZ* family members may be clock regulated.

Materials and Methods

Plant Materials and Growth Conditions

Arabidopsis thaliana seeds were surface sterilized for 3 - 4 h, plated on 1X Murashige and Skoog (MS) medium supplemented with 1.5% sucrose (w/v) and stratified in the dark for 3 nights at 4°C. Plants were grown at constant 22°C with ~90 µmol photons·s-1·m-2, in diurnal light conditions unless otherwise indicated. Columbia-0 (Col-0) was used as the wild-type (WT) control. The *platz2* and *at1g32700* mutants are both SALK T-DNA insertion mutant lines (SALK_016183.53.20 and SALK_208208C) for *PLATZ2* and *AT1G32700*, respectively. Homozygosity for the mutation was confirmed by genotyping, and the reduced expression of *PLATZ2* was confirmed by qRT-PCR. To generate *35S::PLATZ2-OX* transgenic lines, the coding sequence of *PLATZ2* was PCR purified and cloned into pENTR d-TOPO (Invitrogen). LR Clonase II was used to perform the Gateway reaction with the pB7 vector. Sanger sequencing was completed at the Institute for Integrated Genome Biology (IIGB) Genomics Core at University of California, Riverside (UCR) to confirm the sequences. The vectors were transformed into WT plants *via Agrobacterium*-mediated transformation. Two independent T3 lines (*OX-6* and *OX-7*) were selected and confirmed as overexpression lines by qRT-PCR. Two T2 epitope tagged (GFP) transgenic lines, *35S::PLATZ2-GFP*, were similarly created and confirmed by performing the LR reaction with the pEARLY101 vector.

Metadata analysis

Published circadian datasets were analyzed to determine if *PLATZ* family members display rhythmic patterns of expression (Mockler et al., 2007; Wilkins et al., 2010; Romanowski et al., 2020; Bonnot and Nagel, 2021). The transcriptome and translatome heat maps were generated

using CAST-R (Bonnot et al., 2022). Published transcriptome studies examining heat, cold, drought, and hypoxia were analyzed to identify if any *PLATZ* family members respond to any of these stresses (Mustroph et al., 2009; Wilkins et al., 2010; Calixto et al., 2019; Bonnot and Nagel, 2021). Published ChIP-sequencing (ChIP-seq) studies were examined to determine if there is evidence for CCA1, LHY, or TOC1 binding to the promoter regions of any *PLATZ* genes (Huang et al., 2012; Nagel et al., 2015; Adams et al., 2018)

Motif analysis

TAIR's bulk data download tool was used to download the 500 bp upstream of the *PLATZ* genes. Inputting these sequences into the Simple Enrichment Analysis (SEA) tool through the MEME suite resulted in the identification of enriched motif sequences based on the Franco-Zorilla et al 2014 motif database (Bailey et al., 2006; Franco-Zorrilla et al., 2014; Bailey and Grant, 2021).

Heat shock assay

Seeds were sterilized as described above, plated on MS plates with filter paper, and stratified for 3 nights before transfer to diurnal (12h light: 12h dark) conditions at constant 22℃. On day 4, 25 germinating individuals per genotype were transferred to a quadrant of a new square MS plate. On day five, plates were transferred to water baths at 24℃ or 45℃ for 30 min starting at ZT6. Plants were imaged 12 days after heat treatment.

RNA-sequencing set-up and analysis

Three biological replicates of WT (Col-0), *platz2, AT1G32700* (*700*)*,* and *PLATZ2-OX7 (OX7)* were grown for 11 days in diurnal conditions at 22℃. Sampling occurred at 1 hour (Zeitgeber 1; ZT1) and 8 hours (ZT8) after lights on following a 1 hour temperature pulse of 37℃ or maintenance at 22℃. 2 µg of total RNA was isolated (ThermoScientific) and DNAse I treated (Millipore Sigma). mRNA purification and libraries were prepared as previously described (Blair et al., 2019). Final libraries were purified using a 0.8x ratio of Ampure XP beads. Library quantity and quality were confirmed by Qubit 2.0 Fluorescence Reader (ThermoFisher Scientific) and Bioanalyzer 2100 (Agilent Genomics). Sequencing was performed at the UCR IIGB Genomics Core facility on the NextSeq500 (Illumina), which generated single-end 75 bp sequences. Hisat2 was used to map reads to the TAIR10 genome, and DeSeq2 was used to determine differential gene expression (Love et al., 2014; Kim et al., 2015; H Backman and Girke, 2016). Differentially expressed genes (DEGs) were defined as genes with -1 > Log2Fold Change (LFC) > 1 and False Discovery Rate (FDR) < 0.05, and Gene ontology (GO) terms were assigned using a previously published pipeline (Bonnot et al., 2019). See (Blair et al., 2019) for additional analysis pipeline details.

Ortholog identification and phylogeny

Orthologs were identified by reciprocal blast and ortholog groups were identified with Orthofinder (Emms and Kelly, 2019). Phylogenetic trees were assembled using the Multiple Sequence Alignment tool from Clustal Omega (Madeira et al., 2022). These results are supported by established phylogenetic analyses (Holmes, 2017; Wang et al., 2018).

Results

The circadian clock regulates *PLATZ* family members

Previous work indicates that the circadian clock regulates the expression of *PLATZ2* and that *PLATZ2* belongs to the plant-specific *PLATZ* family of transcription factors (Blair et al., 2019; Holmes, 2017). To determine if other *PLATZ* family members are also clock regulated, we performed a metadata analysis using published studies that identified cycling genes (Mockler et al., 2007; Wilkins et al., 2010; Romanowski et al., 2020; Bonnot and Nagel, 2021). Across all four datasets, 6 of the 11 *PLATZ* genes exhibit rhythmic expression and specifically three *PLATZ* genes (*AT5G46710, AT1G32700,* and *PLATZ2)* were identified as circadian regulated in all 5 time course studies (**Figure 3.1A**). To further support clock regulation of the *PLATZ* genes mRNAs, we utilized published ChIP-sequencing (ChIP-seq) studies for critical clock transcription factors, and identified evidence for CCA1, LHY, and TOC1 binding to five *PLATZ* family members (**Figure 3.1A**). Interestingly, both *PLATZ2* and *AT1G21000* (*PLATZ1*) promoters show evidence of binding by three clock proteins, whereas AT5G46710 and AT1G32700 promoters are occupied by only LHY and TOC1, respectively (**Figure 3.1A**). Next, the Simple Enrichment Analysis (SEA) *cis*-element recognition tool (Bailey et al., 2009; Bailey and Grant, 2021) was used to identify the Evening Element (EE; AAATATCT) in the promoter region of five *PLATZ* genes and the G-box (CACGTG) in the promoters of all cycling *PLATZ* genes (**Figure 3.1B**). The EE is associated with CCA1 binding, whereas the G-box is associated with the PRRs (Franco-Zorrilla et al., 2014). In both transcriptome and translatome data from seedlings, *PLATZ* cycling gene transcripts peak in expression during the late afternoon/early evening (Figure 3.1C and 3.1D; Bonnot and Nagel, 2021). The evening expression and presence of the EE in *PLATZ* cycling genes is consistent with alleviation of repression by CCA1 in the evening (Nagel et al., 2015). Biological GO analysis of co-expressed gene transcripts revealed

one enriched term, response to nitrogen compounds (p value = 4.12E-02), among the *PLATZ* family members, which suggests that *PLATZ* genes may have broad functions in cell metabolism. Taken together, this is strong evidence for clock regulation of 3-5 *PLATZ* family genes.

PLATZ **family members respond to abiotic stress**

Arabidopsis PLATZ genes have been previously implicated in abiotic stress responses (González-Morales et al., 2016; Blair et al., 2019; Liu et al., 2020). This is additionally compelling considering the well-documented circadian regulation of the abiotic stress response in conjunction with clock regulation of *PLATZ* family members (Covington et al., 2008; Grundy et al., 2015; Bonnot et al., 2021; **Figure 3.1**). Thus, to determine the extent of *PLATZ* genes function during abiotic stress, we performed a metadata analysis using published transcriptomic studies performed under various stress conditions (**Figure 3.2A**). Preference was given to studies that analyzed a stress at multiple time points, which ultimately resulted in the selection of four studies that investigated the following four abiotic stress responses: heat, cold, drought, and hypoxia (Mustroph et al., 2009; Wilkins et al., 2010; Calixto et al., 2019; Bonnot and Nagel, 2021). Briefly, the Calixto/cold transcriptomic analysis used 5-week-old plants grown in diurnal (12h light: 12h dark) conditions and harvested rosettes (9-13) every 3 hours at 20°C and 4°C (Calixto et al., 2019). The Bonnot/heat analysis used diurnally grown, 13-day-old plants and sampled every 3 hours after 1 hour at 37°C (Bonnot and Nagel, 2021). The Wilkins/drought analysis grew plants in diurnal conditions for 28 days before beginning water withholding (Wilkins et al., 2010). Once 25% of water was lost from stressed plants, rosette tissue was harvested every 6 hours for 24 hours (Wilkins et al., 2010). In the Mustroph/hypoxia study, plants were grown vertically in long day (16h light: 8h dark) conditions for 7 days before exposure to hypoxic conditions (Mustroph et al., 2009). Our analysis of these datasets revealed that *PLATZ* genes respond to temperature stress with 7 of 11 responding to heat stress and 6 of 11 responding

to cold stress with five of these responding to both temperature stresses (**Figure 3.2A**). Five *PLATZ* genes respond to hypoxia stress, and only one gene, *PLATZ1,* responds to drought stress (**Figure 3.2A**). Fittingly, experimental evidence supports a role for *PLATZ1* the drought stress response (González-Morales et al., 2016). Additionally, many of the *PLATZ* genes respond to multiple abiotic stresses with four responding to at least three of the stresses and an additional three responding to two abiotic stress events (**Figure 3.2A**). This indicates that some of the *PLATZ* family members may be generally responsive to abiotic stress, while some are more specific to a certain stress response.

To further determine the extent of *PLATZ* genes regulation during abiotic stress, we used the SEA tool to identify associated abiotic stress motifs (Bailey and Grant, 2021). Strikingly, 10 *PLATZ* genes contain the Heat Shock Element (HSE; AGAANNAAGAAGAAN) providing strong support for the *PLATZ* family participating in the heat stress response (**Figure 3.1B**). Thus, to enhance our understanding of how *PLATZ* genes respond to heat stress, we next investigated how the family members respond to heat over 24 hours. The cycling *PLATZ* genes (4 at the transcriptome and 5 at the translatome level) exhibit upregulation to heat across the 24 hour period (**Figure 3.2B, 3.2C**). The upregulation is slightly higher for the cycling genes at specific time points, such as ZT63 in *AT1G32700*, at the translatome level indicating that the translation of these genes may be required for a rapid heat stress response at certain times of day (**Figure 3.2B, 3.2C, 3.3A**). *AT5G46710* shows a striking profile with very strong upregulation in response to heat at all times of day, and similarly, *AT1G32700* also exhibits upregulation in response to heat across the time course (**Figure 3.3A, 3.3B**). Conversely, *PLATZ2* displays significant upregulation in response to heat at specific timepoints at the transcriptome, ZT48 and ZT66, and the translatome, ZT48, ZT51, ZT63, and ZT69, level, supporting *PLATZ2* transcript abundance

being gated, or responding to heat differently dependent on the time of day the stress is applied, in response to pulses of heat (**Figure 3.3C**).

PLATZ2 may be involved in the plant heat stress response

To gain a better understanding of the functional impact of *PLATZ* genes in the heat stress response, we performed a heat shock assay using SALK T-DNA insertion lines for *PLATZ2* and *AT1G32700*, referred to as *platz2* and *700*, respectively, and a *PLATZ2* overexpression line (*35S::PLATZ2-OX7)*, referred to as *OX-7*, which was confirmed by qRT-PCR (**Figure 3.4A-3.4C**). Seedlings, which were developmentally indistinguishable at this age, were grown at 22°C in diurnal conditions for five days before exposure at ZT6 to a control 22°C or an experimental 45°C water bath for 30 minutes (**Figure 3.4D**). The 30-minute heat stress revealed a trend for increased heat susceptibility in the overexpression line although this is not significant (**Figure 3.4E and 3.4F**). The 30-minute heat treatment was insufficient to show any survival phenotype differences (**Figure 3.4F**). Thus, future experiments should include a 45-minute and 1 hour treatment. Additional genotypes, including a second mutant allele for *platz2*, *OX-6*, and other *PLATZ* family members should also be included in future assays.

Some *PLATZ* family members have been shown to be nuclear localized including *PLATZ2* (So et al., 2015; Kim et al., 2018; Fu et al., 2020; Liu et al., 2020). Additionally, ChIP qRT-PCR demonstrates PLATZ2 binding to the *CBL4/SOS3* and *CBL10/SCaBP8* promoters; both *CBL4/SOS3* and *CBL10/SCaBP8* are involved in the salt overly sensitive (SOS) pathway and are induced by salt stress (Liu et al., 2020). To determine the broader transcriptional effect of *PLATZ2* during heat, future work should include a ChIP-seq study to see what promoter regions PLATZ2 occupies and to determine if that is altered in warmer temperature. To do this requires the characterization of GFP or epitome tagged *PLATZ2* lines. GFP fluorescence analysis of

seedlings of the lines generated indicates that *35S::PLATZ2.1-GFP* and *35S::PLATZ2.1-GFP*, have functional tags, although this should be confirmed and quantified by western blot for these lines (**Figure 3.5**).

To better understand the broader impact of *PLATZ2* during heat stress, we performed an RNA-seq experiment using WT, *platz2*, *OX-7*, and *700* on 11-day old seedlings grown in diurnal conditions and sampled at ZT1 and ZT8 following a 1 hour temperature pulse of 37°C or maintenance at 22°C. Differential expression analysis examining each *PLATZ* genotype to the WT revealed a greater number of DEGs during heat stress as compared to the ambient temperature at both ZT1 and ZT8 (**Figure 3.6A and 3.6B**). In the morning, the greatest perturbation of the transcriptome occurred at 37°C in *OX-7* compared to the WT with 217 DEGs (**Figure 3.6A**). In contrast, the same comparison at 22°C only resulted in 6 DEGs (**Figure 3.6A**). While cluster 1 was not enriched for any Gene Ontology (GO) terms, cluster 2 was enriched for terms associated with reproductive system development (**Supplemental Table 1**). As many genes in cluster 2 demonstrate downregulation during ambient temperature and upregulation during heat stress, the enrichment for reproductive system development is consistent with earlier flowering to avoid a heat stress event (**Figure 3.6A**). In the evening, there are more DEGs than in the morning, suggesting that the *PLATZ* genes have a greater impact on the transcriptome at the time point where heat stress is more physiologically relevant (**Figure 3.6A and 3.6B).** DEGs in cluster 2 are downregulated at both 22°C and 37°C suggesting these genes may be regulated by *PLATZ2* and *AT1G32700* independent of temperature (**Figure 3.6B)**. These genes are enriched for defense, response to abiotic stress, and multiple metabolic processes GO biological process terms (**Supplemental Table 1**). Cluster 4 contains genes that are upregulated in response to heat stress and are enriched for RNA processing and cell wall organization GO terms (**Figure 3.6B, Supplemental Table 1**). To determine, which genes are regulated by *PLATZ* gene misexpression

rather than the heat stress itself, we next compared the morning and evening DEGs to heat stress DEGs in the WT resulting in the identification of numerous genes that are controlled by *PLATZ2* and *AT1G32700* both independently during heat stress and in conjunction with the heat stress. (**Figure 3.6C and 3.6D**).

Rice *PLATZ* orthologs cycle and 1 sorghum *PLATZ* ortholog is heat-responsive

Little is known about how *PLATZ* family members are clock-regulated or heat-responsive outside of Arabidopsis, so next, we investigated whether *PLATZ* gene transcript abundance is regulated by high temperature in crop species, we identified *PLATZ* orthologs in sorghum and rice via reciprocal blast and confirmed the orthologous relationship using Orthofinder (Emms and Kelly, 2019). Then, we examined heat-responsiveness of the 11 sorghum *PLATZ* (*SbPLATZ*) orthologs using an unpublished sorghum transcriptome dataset from our lab, which applied a 42°C, 1 hour heat treatment at 4 time points (1, 6, 9, and 15 hours after dawn) in two sorghum accessions (heat susceptible, RTX430 (RTX), and heat tolerant, Macia) (**Figure 3.7A**). Plants were grown in diurnal light conditions at 30°C and 22°C during the day and night periods, respectively. In this experiment, the mRNA of one *SbPLATZ* ortholog, *Sobic.004G275200*, was identified as significantly upregulated during heat stress at all time points evaluated in both genotypes except for Macia at ZT6, indicating a time of day response for this gene (**Figure 3.7B and 3.7C**). One explanation for this could be that lower *Sobic.004G275200* transcript levels at ZT6, when heat stress is likely to occur, may confer some level of heat tolerance. Gene Ontology (GO) analysis demonstrated that genes upregulated in response to heat are enriched for heat stress associated terms, while genes downregulated in response to heat stress are enriched for photosynthesis, response to light, cell wall modification, nucleosome assembly, and metabolic processes (Blair et al., 2019). Future experiments to measure heat tolerance in Sobic.*004G275200* mutants generated by CRISPR-CAS9 directed mutagenesis in the RTX and Macia backgrounds could be helpful to

determine if *Sobic.004G275200* plays an important role in conferring thermotolerance. Interestingly, Sobic.004G275200 is also the only *SbPLATZ* gene which exhibits circadian rhythmicity (Lai et al., 2020).

Next, we utilized known phylogenetic analyses and reciprocal blast to identify rice *Platz* (*OsPLATZ*) orthologs in conjunction with rhythmicity data from the DIURNAL database and found evidence that 16 of the *OsPLATZ* orthologs exhibit rhythmicity. In diurnal (LDHH) conditions, 4 *OsPLATZ* orthologs cycle with peak expression at ZT20 and 1 ortholog has peak expression at ZT0 (**Figure 3.7D**). In plants exposed to constant light after entrainment in diurnal conditions (LLHH_LDHH), the timing of peak expression is shifted for some of the *OsPLATZ* orthologs (**Figure 3.7D and 3.7E**). For example, LOC_Os06g41930 (lavender line) peaks at ZT20 in diurnal conditions and at ZT12 in constant light (**Figure 3.7D and 3.7E**). However, other genes such as LOC_Os04g50120 (red line) peaks at ZT20 in both growth conditions (**Figure 3.7D and 3.7E**). These data indicate that some of the *OsPLATZ* orthologs may be regulated by the circadian clock. Future work should investigate how *OsPLATZ* genes respond during heat stress and if heat stress perturbs their circadian rhythms.

Discussion

To our knowledge, the *PLATZ* family has not been studied in relation to the circadian clock besides our previous work in *Arabidopsis* (Chapter 1; (Blair et al., 2019) or in other species. To more clearly elucidate circadian regulation of the *PLATZ* family, we leveraged published studies and bioinformatics tools to show evidence for clock regulation of 5 *PLATZ* genes (**Figure 3.1**). In particular, there is strong support for circadian regulation of *PLATZ2*, which showed rhythmic expression in five circadian datasets with peak expression in the early evening, occupation by

CCA1, LHY, and TOC1 according to ChIP-seq datasets, and both an EE and CBS in its promoter region (**Figure 3.1**).

Across plant species, *PLATZ* family members have previously been shown to respond to abiotic stresses including drought, NaCl, ABA, and hormones (So et al., 2015; González-Morales et al., 2016; Kim et al., 2018; Liu et al., 2020; Li et al., 2022). In *Arabidopsis*, work on *PLATZ1* and *PLATZ2* revealed their regulation in response to to salt and drought (González-Morales et al., 2016; Liu et al., 2020). In the *cca1lhy* double mutant, *PLATZ2* responds to heat stress in the morning, but otherwise little is known about *PLATZ* family members during temperature stress (Blair et al., 2019). Here, we used a meta-data approach to identify which PLATZ family members respond to abiotic stress including heat stress, cold stress, hypoxia, and drought. We found that of the 11 *PLATZ* genes seven respond to heat stress, six respond to cold stress, one responds to drought, and five respond to hypoxia (**Figure 3.2**). Of the cycling *PLATZ* family members, all are induced in response to heat stress, but some genes, *AT1G32700* and *AT5G46710,* are constitutively heat induced, while *PLATZ2* shows a time of day dependent response to heat (**Figure 3.3**).

There have been several phylogenetic analyses of the *PLATZ* family in *Arabidopsis* and other species, but less work has been done on the functional characterization of the family members, especially in the heat stress response (Holmes, 2017; Wang et al., 2018; Azim et al., 2020; Fu et al., 2020; He et al., 2021; Li et al., 2022; Zhao et al., 2022). Therefore, we performed a heat shock assay using *Arabidopsis* genotypes, which provided preliminary evidence that *PLATZ2* ectopic expression may enhance susceptibility to heat stress (**Figure 3.4**). While the heat shock assay did not show a significant difference in survival, it is likely that the heat shock was too mild for a noticeable difference. Therefore, future assays should be conducted at 45 and 60 min time intervals to more robustly assess survival after heat shock. Next, we performed a

transcriptome analysis to elucidate the larger impact of *PLATZ2* during heat shock. Differential expression analysis identified that *PLATZ* genes have a greater impact in the late afternoon as compared to the morning, which coincides with timing of typical heat stress events in the afternoon (**Figure 3.6**). Future analysis should be conducted to more thoroughly analyze these transcriptome datasets and more clearly identify the downstream impact of *PLATZ* genes.

Lastly, we investigated the *PLATZ* orthologs in sorghum and rice to determine if clock regulation and heat responsiveness is conserved across these species. We found that ~44% and ~56% of *OsPLATZ* genes exhibit a rhythmic oscillation pattern in diurnal and constant conditions, respectively, which is the first example showing that *OsPLATZ* genes are clock regulated (**Figure 3.7**). In sorghum, one ortholog, *Sobic.004G275200*, shows rhythmic oscillation and responds to heat stress by time of day (**Figure 3.7**).

To improve our understanding of the function of PLATZ family members, additional characterization should be completed with future experiments. For example, future work should investigate *PLATZ* family members in other species to determine if circadian regulation and heat-responsiveness is conserved. Additionally, studies in maize and rice demonstrate that some PLATZ family members proteins interact with components of RNA Pol III (TCP1 and RPC53) suggesting that the *PLATZ* family may be involved with small-noncoding RNA transcription to regulate the cell cycle and cell growth (Li et al., 2017; Wang et al., 2018). According to published phylogenetic analysis (Wang et al., 2018), PLATZ2's most closely related maize ortholog associates with TCP1 and RPC53 (Wang et al., 2018). Future yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) studies should investigate conservation of protein-protein interaction of PLATZ2 and other PLATZ transcription factors in *Arabidopsis*.

Future work should also prioritize assessing the response to heat stress in the other cycling *PLATZ* family members, especially *PLATZ1* and *AT5G46710* by repeating the heat shock assay and transcriptomic analysis with mutants and overexpression lines for these genes. Cloning is underway for *PLATZ1* overexpression and CRISPR mutant lines for *PLATZ1*. While there is a SALK T-DNA insertion line available for *AT5G46710*, our qRT-PCR results indicate that there is no reduced expression in this line (data not shown). Therefore, we excluded the *AT5G46710* mutant from our transcriptome study. Additionally, we measured plant survival in response to heat shock after heat acclimation at 28°C, but preliminary results indicate that the *PLATZ2* mutant and ectopic overexpression lines do not show a difference in survival (data not shown). Protocol optimization and additional replication should be done in the future to assess survival more definitively after heat acclimation.

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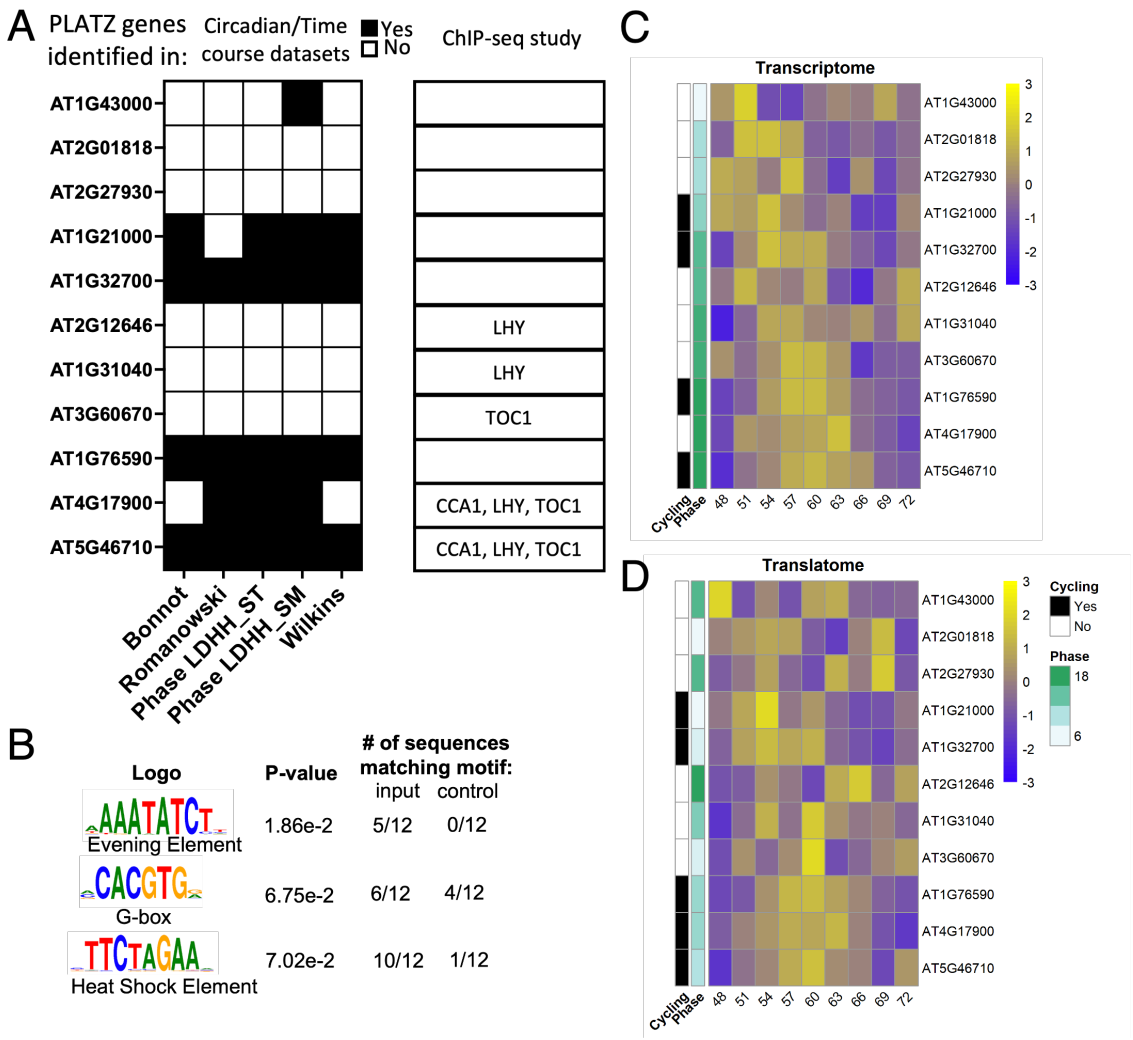
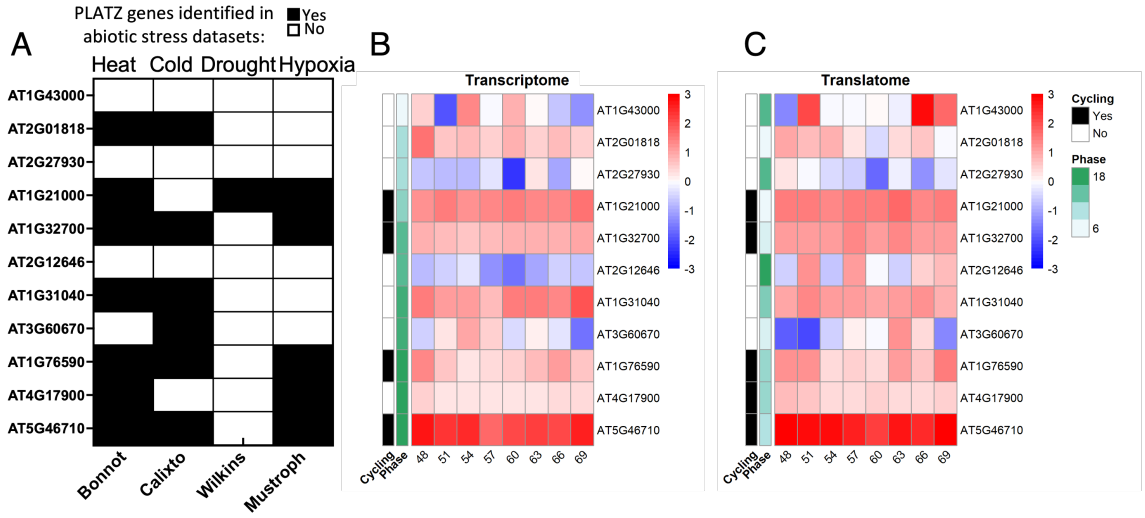
Figures

Figure 3.1: The clock regulates *PLATZ* family members.



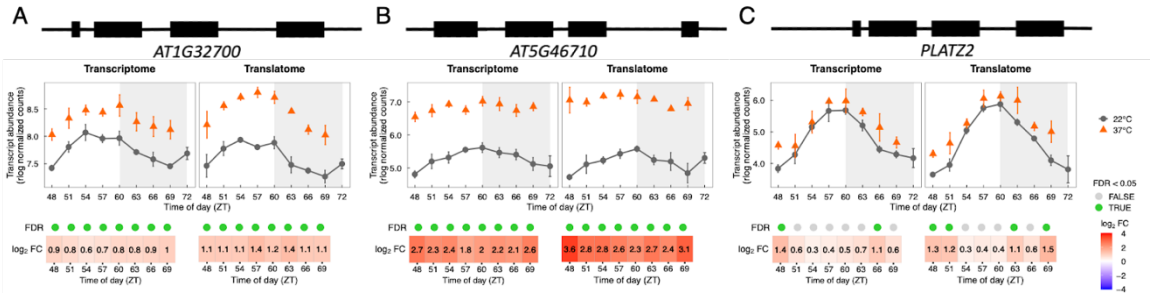
(A) *PLATZ* gene mRNAs that cycle in five different circadian datasets are denoted as cycling or not cycling with a black or white box, respectively (Mockler et al., 2007; Wilkins et al., 2010; Romanowski et al., 2020; Bonnot and Nagel, 2021). ChIP-seq study column indicates which clock transcription factors occupy the promoter region of a given *PLATZ* gene based on published ChIP-sequencing studies (Huang et al., 2012; Nagel et al., 2015; Adams et al., 2018). (B) Simple Enrichment Analysis (SEA) analysis was conducted with default settings using the Franco-Zorilla et al 2014 PBM motifs as input (Franco-Zorrilla et al., 2014). The transformed expression values, measured by rlog (regularized-logarithm) normalized counts, in the (C) transcriptome and (D) translatome are shown on a color scale from yellow to blue every 3 hours over 24 hours for the *PLATZ* family members plotted using the CAST-R database (Bonnot et al., 2022). Cycling and non-cycling are denoted by a black and white box, respectively. The phase or timing of peak expression is shown on a green scale with ZT0 corresponding to white and ZT18 corresponding to dark green.

Figure 3.2: *PLATZ* family member transcripts accumulate in response to heat and other abiotic stresses.



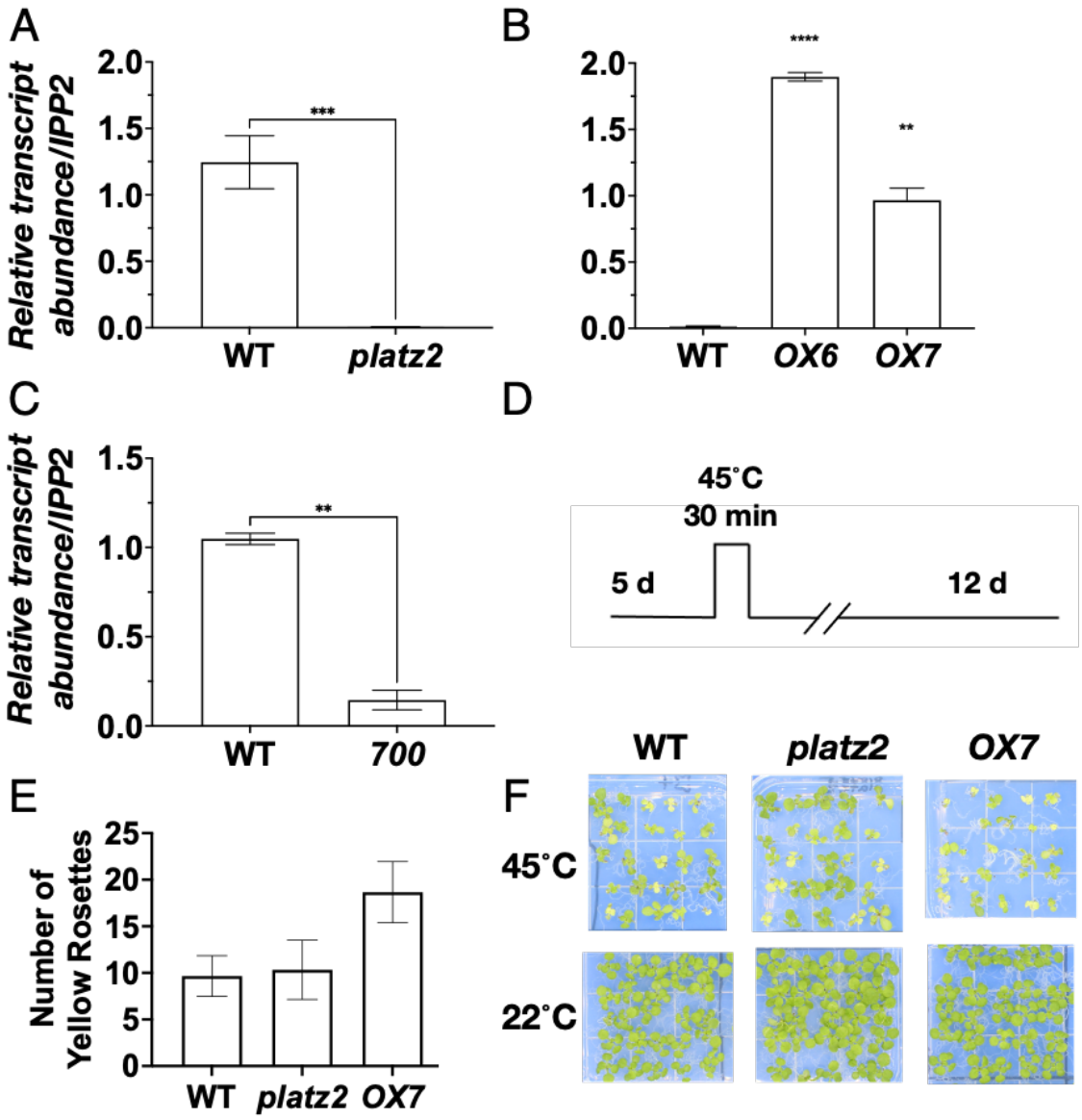
(A) *PLATZ* gene mRNAs that respond to heat, cold, drought, and hypoxia are denoted as stress-responsive with a black or white box, respectively (Mustroph et al., 2009; Wilkins et al., 2010; Calixto et al., 2018; Bonnot and Nagel, 2021). Log2 Fold Change (LFC) values of 37°C compared to 22°C at the (B) transcriptome and (C) translatome are shown on a color scale from red (up-regulated in response to heat) to blue (down-regulated in response to heat) every 3 hours over 24 hours for the *PLATZ* family members plotted using the CAST-R database (Bonnot et al., 2022). Cycling and non-cycling are denoted by a black and white box, respectively. The phase or timing of peak expression is shown on a green scale with ZT0 corresponding to white and ZT18 corresponding to dark green.

Figure 3.3: *PLATZ* genes are upregulated in response to heat at the transcriptome and translatome level.



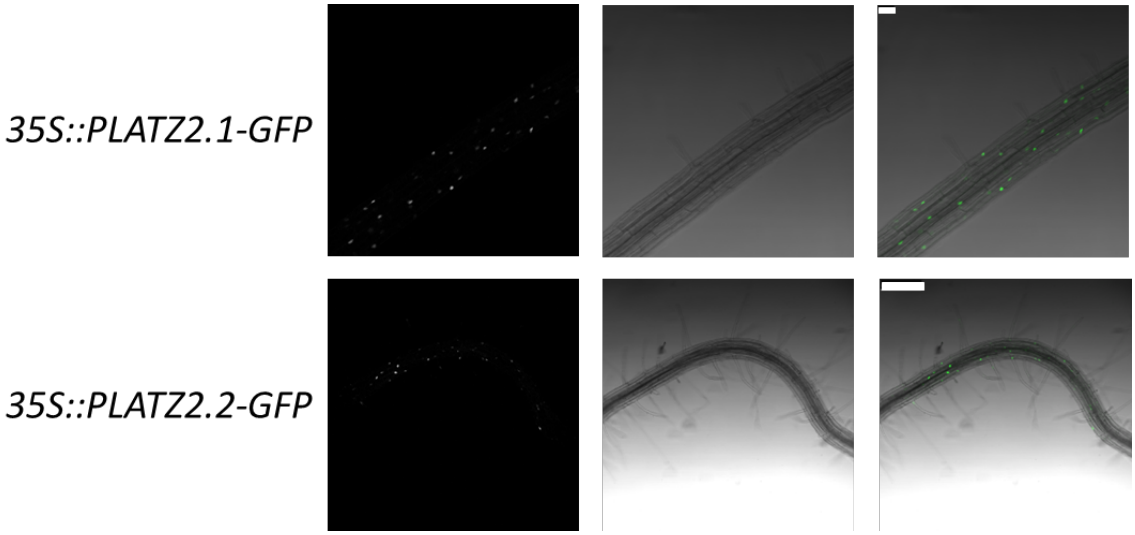
Transcript abundance was plotted using the CAST-R database to show the transcript abundance of (A) *AT1G32700*, (B) *AT5G46710*, and (C) *AT1G76590/PLATZ2* over 24 hours at 22°C and after 1 hour temperature pulse of 37°C (Bonnot et al., 2022). Log2 Fold Change (LFC) values are shown on a scale of red to blue with green circle indicating a significant False Discovery Rate (FDR). Gene models for (D) *AT1G32700*, (E) *AT5G46710*, and (F) *AT1G76590/PLATZ2* are based on Araport11.

Figure 3.4: *PLATZ2* may contribute to the heat stress response.



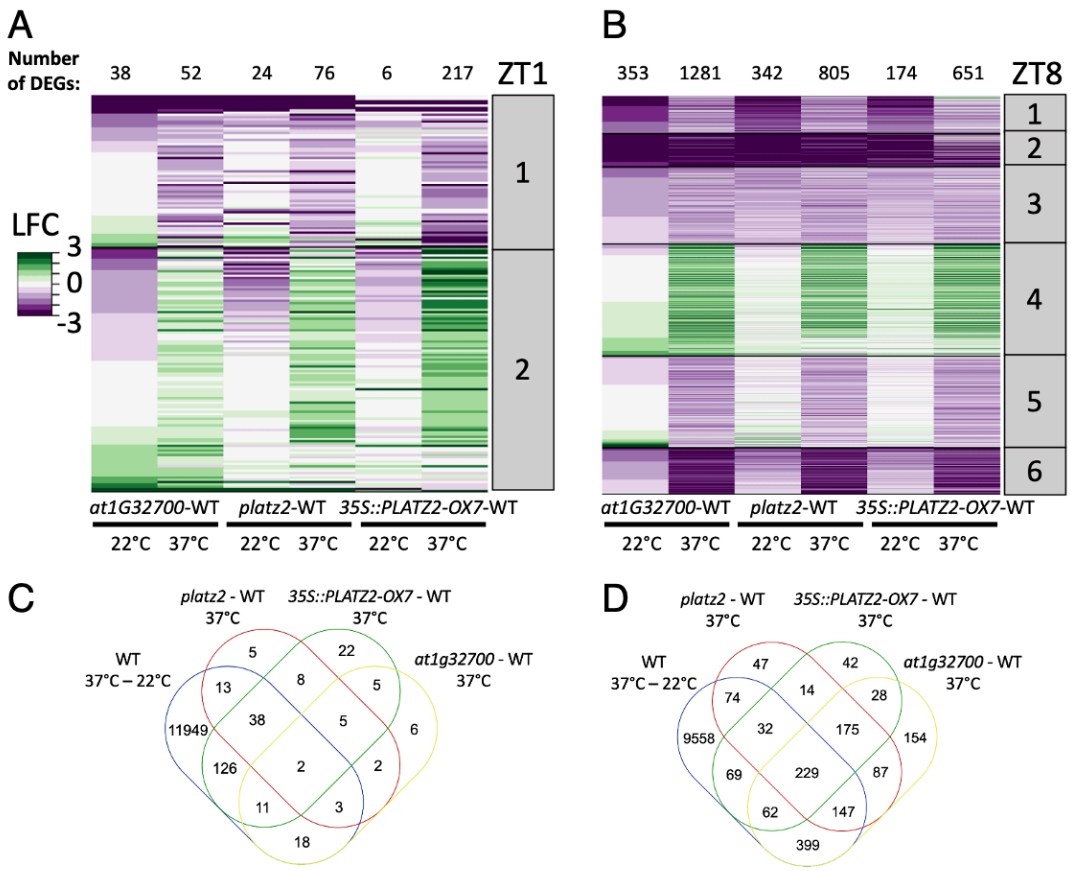
qRT-PCR of *PLATZ2* transcript accumulation in 8-day-old seedlings grown in diurnal conditions and sampled at (A) ZT8 for platz2, (B) ZT0 for *35S::PLATZ2-OX6* and *35S::PLATZ2-OX7*, and (C) ZT8 for *AT1G32700*. mRNA levels are normalized to *IPP2* (mean values ± SE, n=3, ***P ≤ 0.001; **P ≤ 0.01; *P ≤ 0.05, unpaired student t-test). (D) Experimental set-up for heat shock assay in which plants were grown for five days at 22C before exposure to 45C water bath for 30 min starting at ZT6. (E) The number of yellow rosettes was plotted to indicate plant response to the heat shock and (F) images were taken 12 days post heat shock.

Figure 3.5: Independent *PLATZ2* overexpression lines display GFP fluorescence in nuclei.



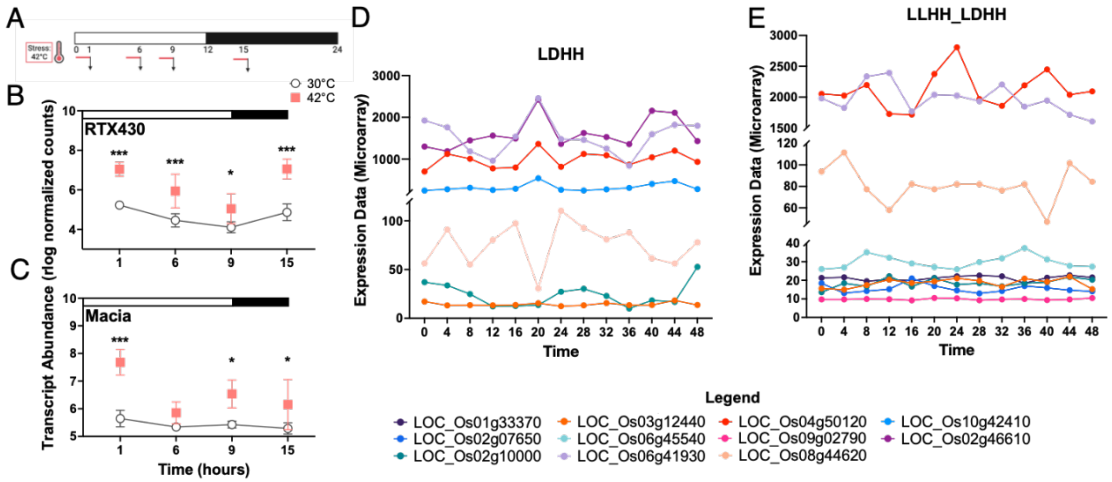
GFP-fluorescent images of five-day-old Arabidopsis roots for two T2 *35S::PLATZ2-GFP* transgenic lines. The top three images have white scale bar equivalent to 50 µm and the bottom three images to 250 µm.

Figure 3.6: *PLATZ2* and *AT1G32700* have a greater impact on the transcriptome in the evening and during heat stress.



Transcriptomic analysis was performed using 11-day-old, diurnally grown WT, *platz2*, *at1g32700*, and *35S::PLATZ2-OX7* seedlings after a 1 hour 37˚C temperature pulse performed at (A,C) ZT0 and (B,D) ZT7. (A,B) DEGs were selected by -1 < Log2 fold change (LFC) < 1 and false discovery rate (FDR) ≤ 0.05. DEGs specific to the heat response vs. *PLATZ2* mis regulation during heat stress at (C) ZT1 and (D) ZT8.

Figure 3.7: *PLATZ* rice genes cycle and 1 *PLATZ* sorghum gene is heat-responsive.



Survey of *PLATZ* gene regulation in sorghum and rice. (A) Heat susceptible sorghum, RTX430, and heat tolerant sorghum, Macia, accessions were sampled at hours 1, 6, 9, and 15 after a 1-hour temperature pulse of 42˚C. Transformed expression values measured by rlog (regularized-logarithm) normalized counts of *Sobic.004G275200* in (B) RTX430 and (C) Macia at each time point (rlog ± SD, n=3, ***P ≤ 0.0001, **P ≤ 0.001, **P ≤ 0.01; FDR 30˚C-42˚C). Cyclic pattern of expression for rice *PLATZ* orthologs in (D) diurnal and (E) constant conditions after entrainment in diurnal.

Conclusion and Future Directions

Conclusion

This dissertation expands current knowledge regarding the downstream, temperature-responsive components of the circadian clock regulatory network in context of time of day. In my first chapter, I identified the *Arabidopsis* clock regulated transcriptome dynamics after temperature stress in *cca1lhy* and *prr7prr9* following a 1 hour cold (10°C) or heat (37°C) pulse at ZT1 and ZT6. My results indicate that the magnitude and occurrence of the temperature stress response is highly dependent on the time of day that the stress is applied at the transcriptome level. I also identified a time of day dependent and clock regulated subset of genes that included the cold and heat responsive transcription factors, *CDF6* and *PLATZ2*, respectively. In my second chapter, I investigated how *CDF6* is clock regulated during cold stress to finetune plant development. Specifically, the clock through *CCA1* gates the transcript accumulation of *CDF6* across the 24 hour period during 10°C pulses of cold stress. Ectopic expression of *CDF6* in the vasculature results in delayed germination at ambient temperature and delayed flowering in long day conditions in both ambient and cold temperature. Expression analysis indicates that vasculature expressed *CDF6* reduces the transcript abundance of *FT, BFT,* and *CO* at ambient temperature and to a greater extent under cold stress. RNA-sequencing analysis further showed that *CDF6* has a greater impact on the transcriptome during cold stress than it does during ambient temperature. In my third chapter, I investigated the *PLATZ* family of plant-specific transcription factors during heat stress. Approximately 50% of *Arabidopsis PLATZ* genes are clock regulated and heat responsive. Some of the *PLATZ* family members, such as *AT1G32700* and *AT5G43710*, are constitutively induced in response to heat stress, while others, such as *PLATZ2* have a time of day dependent heat induction. Many phylogenetic studies of the *PLATZ* family members have been performed for various species, such as maize, wheat, sorghum, and soybean (Holmes, 2017). I

utilized these to investigate presumed rice and sorghum *PLATZ* orthologs to determine if these genes are heat responsive or clock regulated. Transcriptomic studies indicate that one sorghum ortholog displays cyclic expression and is heat responsive, while about ~50% of the rice *PLATZ* orthologs show rhythmic expression.

In this dissertation, I identified clock controlled and temperature responsive genes at the transcriptome level, and I characterized *CDF6* and *PLATZ2* to more closely examine how the clock differentially regulates plant growth and development to tightly control the plant cold and heat stress response, respectively. Overall, this work highlights the importance of studying the circadian clock in context of time of day to deduce the impact of the temperature stress response.

Future Directions

While this dissertation examines clock regulation of the plant temperature stress response at multiple scales, there are various experiments that could be done in the future to strengthen and expand our comprehensive understanding of the clock regulated temperature responsive network. Firstly, the work described in my first chapter continues to be a source for hypothesis generation to understand clock regulation of the temperature stress response, especially when considered in conjunction with another study from our lab that performed a heat stress time course (Bonnot and Nagel, 2021). Secondly, the work described in my second chapter focused largely on expression analysis to show *CCA1* regulates *CDF6* which in turn regulates *FT* during the cold. Due to the function of *CCA1* and the *CDFs* as transcription factors, it is possible that the molecular mechanism of control for the *CCA1-CDF6-FT* regulatory module would be direct binding of the transcription factor to each target gene's promoter region, which could be addressed with ChIP qPCR performed under both cold and ambient temperatures. Additionally, I think considering these results with the RNA-sequencing dataset and any chromatin accessibility experiments performed under cold and ambient conditions could also be an insightful addition to the second

chapter work. Thirdly, the work in my third chapter investigated the function of the *PLATZ* family of transcription factors mostly in *Arabidopsis* and to a lesser extent in rice and sorghum. This preliminary study could be further strengthened by extending the analysis to other species. The *PLATZ* family has orthologs in many species including some photosynthetic algae, such as *Chlamydomonas*; therefore, it would be interesting to determine if there is evidence for circadian regulation of this family in *Chlamydomonas* and other species (Holmes, 2017). Many *Arabidopsis PLATZ* family members that appear to be clock regulated are also induced by heat so it would also be interesting to determine the extent of the conservation of the *PLATZ* heat stress response across species. Future work should also expand on the heat shock assay and transcriptome analysis to include other *PLATZ* mutants and overexpression lines with a focus on those *PLATZ* genes that are clock regulated based on their rhythmic oscillation. The heat shock assay should also be further optimized to include longer periods of stress so that survival can more easily and reproducibly be assessed. While preliminary results showed no difference in survival among *PLATZ* genotypes that were stressed after heat acclimation, this could also be reassessed in the future by testing survival after both a gradual and step-wise acclimatation (Larkindale and Vierling, 2008; Song *et al.*, 2012). Interestingly, some maize and rice *PLATZ* family members bind to components of RNA Pol III, which transcribes 5S rRNA, tRNAs, and some small RNAs (Johnson *et al.*, 2016; Dieci *et al.*, 2007). Future work should investigate how *PLATZ2* and its *Arabidopsis* orthologs may associate with RNA Pol III components to control transcription of small RNAs, especially to determine if *PLATZ* transcription factors regulate transcription of long non-coding RNAs (lncRNAs), which are known to play a role in abiotic stress responses similar to multiple *PLATZ* family members (Jha *et al.*, 2020). A CRISPR approach to create higher order mutants may also be helpful for future work especially to determine any functional redundancies among *PLATZ* family members. Additionally, engineering the expression of *PLATZ* family

members with various cell-type specific promoters may also yield interesting results that could more specifically impact plant growth or development during heat stress.

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